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DEVELOPMENT OF HAEMATOPOIETIC STEM CELLS IN THE HUMAN EMBRYO

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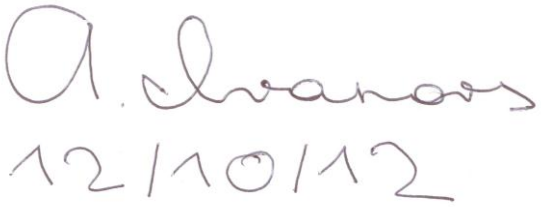
AUTHORSHIP DECLARATION

I hereby declare that this thesis is my own work, except where otherwise stated, and that none of the parts of this thesis has been previously submitted in any form for a degree or any other qualification at this University or any other institution. Information derived from the published or unpublished work of others has been thoroughly acknowledged and referenced.



DEDICATION

I would like to dedicate this work to my beloved grandmother, Natalya Vladimirovna Kovalyova. Бабушка, большое Тебе спасибо за всё!

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I wish to express my deep and sincere gratitude to my supervisor Professor Alexander Medvinsky for all his support and guidance throughout my PhD studies. His genuine passion for science was and still remains an inspiration for me! I thank my second supervisor Professor Marc L. Turner for his useful opinions and advice on the present study. I am in debt to my mentor Dr Stanislav Rybtsov for scientific teaching, extreme patience, many invaluable discussions we had on the project and beyond and his friendship.

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ABSTRACT

Haematopoietic stem cells (HSCs) emerge during embryogenesis and maintain hematopoiesis in the adult organism. Qualitative and quantitative assessment of HSCs can only be performed functionally using the *in vivo* long-term repopulation assay. Due to the lack of such data, little is known about the development of HSCs in the human embryo, which is a prerequisite for the development of new therapeutic strategies. Employing the xenotransplantation assay, I have performed here the spatio-temporal mapping of HSC activity within the human embryo and have shown that human HSCs emerge first in the aorta-gonad-mesonephros (AGM) region, specifically in the ventral wall of the dorsal aorta, and only later appear in the yolk sac, liver and placenta. Human AGM region HSCs transplanted into immunodeficient mice provide long-term high-level multilineage haematopoietic repopulation. These cells, although present in the AGM region in low numbers, exhibit a very high self-renewal potential. A single HSC derived from the AGM region generates around 600 daughter HSCs in primary recipient mice, which disseminate throughout the entire recipient bone marrow and are retransplantable. These findings highlight the vast regenerative potential of the earliest human HSCs and set a new standard for *in vitro* generation of HSCs from pluripotent stem cells for the purpose of regenerative medicine. I have also established a preliminary immunophenotype of the earliest human HSC. These data will be useful for my future studies on the mechanisms underlying the high potency of human embryonic HSCs and on the characterisation of embryonic HSC niche.

LIST OF ABBREVIATIONS

7-AAD – 7-amino-actinomycin

AGM region – the aorta-gonad-mesonephros region

ALDH – aldehyde dehydrogenase

AoD – the dorsal domain of the dorsal aorta

AoV – the ventral domain of the dorsal aorta

BFU-E – burst-forming unit erythroid

CAFC – cobblestone area-forming cell

CFSE – carboxyfluorescein diacetate succinimidyl ester

CFU-C – colony-forming unit-culture

CFU-G – colony-forming unit-granulocyte

CFU-GEMM – colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte

CFU-GM – colony-forming unit-granulocyte, macrophage

CFU-M – colony-forming unit-macrophage

CFU-S – colony-forming unit-spleen

CI – confidence interval

CLP – common lymphoid progenitor

CMP – common myeloid progenitor

CS – Carnegie stage

DPBS – Dulbecco's phosphate buffered saline

ES cell – embryonic stem cell

FACS – fluorescence-activated cell sorting

FBS – foetal bovine serum

GMP – granulocyte and monocyte progenitor

hpf – hours post fertilisation

HSC – haematopoietic stem cell

LTC-IC – long-term culture-initiating cell

Ly5.1/Ly5.1 mouse – B6.SJL-*Ptprc*^{a} *Pepc*^{b}/Boy mouse

Ly5.2/Ly5.2 mouse – C57BL/6 mouse

MEP – megakaryocyte and erythrocyte progenitor

MLP – multilymphoid progenitor

MPP – multipotent progenitor

NK cell – natural killer cell

NOD/SCID mouse – NOD.CB17-*Prkdc*^{scid} mouse

NSG mouse – NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} mouse

SCID – severe combined immunodeficiency

SD – standard deviation

STR analysis – short tandem repeat analysis

UGR – urogenital ridge

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1. INTRODUCTION

1.1. General Introduction

Due to a very limited access to early human embryonic tissues, the embryonic development of the human haematopoietic system remains a largely unexplored area of research. A better understanding of this process is not only of academic interest but also of potential practical importance. Although bone marrow and umbilical cord blood-derived HSC transplantation is performed for a number of therapeutic indications in the clinic, the availability of suitable donors is inadequate. Therefore, many research laboratories are investigating the possibility of generating HSCs under controlled conditions in the laboratory. Embryonic and induced pluripotent stem cells can differentiate *in vitro* into the majority of cell types, including various haematopoietic cells. Theoretically, pluripotent stem cells could become an unlimited source of customised HSCs suitable for clinical application (reviewed by Kaufman, 2009). However, the generation of transplantable HSCs has not been achieved so far. A better understanding of the embryonic development of human HSCs may be instrumental in developing novel protocols for their generation in the laboratory since without a deep understanding of the process *in vivo* it is hardly possible to replicate it *in vitro.*

Although in vertebrate embryos the first haematopoietic cells, primitive erythroblasts, emerge in the yolk sac (Moore and Metcalf, 1970), a growing body of evidence suggests that the AGM region plays a key role in the generation of adult-type or the so-called definitive HSCs (Boisset *et al.*, 2010; de Bruijn *et al.*, 2002; Dieterlen-Lievre, 1975; Medvinsky and Dzierzak, 1996; Medvinsky *et al.*, 1993;

Muller *et al.*, 1994; Zovein *et al.*, 2008), possibly through the endothelio-haematopoietic transition of endothelial cells in the dorsal aorta, which has been most convincingly shown in the zebrafish embryo employing *in vivo* imaging techniques (Bertrand *et al.*, 2010; Kissa and Herbomel, 2010). The mouse AGM region is capable of autonomous initiation and expansion of HSCs (Cumano *et al.*, 2001; Medvinsky and Dzierzak, 1996; Taoudi *et al.*, 2008). The early umbilical cord and the placenta are also involved in HSC development (de Bruijn *et al.*, 2000; Gekas *et al.*, 2005; Ottersbach and Dzierzak, 2005; Robin *et al.*, 2009). Since in the mouse embryo the first definitive HSCs are detected at approximately the same time in different embryonic locations (de Bruijn *et al.*, 2000; Gekas *et al.*, 2005; Kumaravelu *et al.*, 2002; Muller *et al.*, 1994; Ottersbach and Dzierzak, 2005), their primary origin still remains a debatable issue (reviewd by Medvinsky *et al.*, 2011).

The generation of the first immunodeficient mice capable of accepting human tissue grafts (McCune *et al.*, 1988) gave rise to studies on human HSCs, which were usually sourced from the adult bone marrow and the umbilical cord blood (Conneally *et al.*, 1997; Larochelle *et al.*, 1996; Wang *et al.*, 1997). Human HSCs at early embryonic stages were detected only in the liver (Oberlin *et al.*, 2010) and the placenta (Robin *et al.*, 2009). Since human AGM and yolk sac cells have never been tested for HSC activity, it is not clear whether the previous observations in the mouse embryo can be extended to the human embryo. Therefore, considering the potential practical relevance to regenerative medicine, a detailed characterisation of the development of HSCs in the human embryo is the major scientific question addressed by the present thesis.

1.2. Central Terms of This Thesis

HSC is a multipotent stem cell that emerges early during embryogenesis and maintains haematopoiesis during the entire lifespan of the organism through continuous self-renewal and differentiation. It is considered that fully-developed adult-type or the so-called definitive HSCs are capable of long-term high-level haematopoietic repopulation upon direct transplantation into preconditioned adult wild-type (immunocompetent) recipients (reviewed by Medvinsky *et al.*, 2011). Therefore, qualitative and quantitative assessment of definitive HSCs can only be performed *in vivo* using the long-term repopulation assay (Jones *et al.*, 1990; Ploemacher and Brons, 1989; Spangrude *et al.*, 1988; Szilvassy *et al.*, 1990). It should be noted that when discussing mouse embryonic HSCs I use the term 'definitive'; however, to be formal, I omit using this term for human embryonic HSCs since they were tested by transplantation into irradiated adult immunodeficient mice. In theory, the ideal recipient to test the definitive status of human embryonic HSCs would be a preconditioned adult human recipient.

Pre-HSC is a haematopoietic precursor cell in the embryo that precedes the formation of definitive HSCs. Pre-HSCs, unlike definitive HSCs, are not yet mature enough to provide haematopoietic repopulation upon direct transplantation into irradiated adult wild-type recipients (reviewed by Medvinsky *et al.*, 2011). These cells can mature into definitive HSCs *in vitro* (Rybtsov *et al.*, 2011; Taoudi *et al.*, 2008) and can possibly engraft preconditioned neonatal (Ferkowicz *et al.*, 2003; Yoder *et al.*, 1997) and adult immunodeficient mice (Bertrand *et al.*, 2005).

The AGM region is an intraembryonic tissue consisting of the dorsal aorta and urogenital ridges (UGRs). Data obtained in the mouse embryo suggest that the AGM region is the site where the first definitive HSCs emerge. The AGM region originates from the para-aortic splanchnopleura, which combines the dorsal aorta, vitelline artery (also called the omphalomesenteric artery), gut and splanchnopleura (reviewed by Medvinsky *et al.*, 2011).

The yolk sac is a membranous extra-embryonic tissue consisting of extra-embryonic endoderm and mesoderm. In various vertebrate embryos, the differentiation and production of the first primitive nucleated erythroblasts and myeloid cells occur in the yolk sac. Therefore, it was long considered that the yolk sac also produces definitive HSCs. However, the current consensus is that it is more likely that this role belongs to the AGM region (reviewed by Medvinsky *et al.*, 2011).

Primitive haematopoiesis is the first wave of haematopoiesis. It originates in the yolk sac and is characterised by a transient production of primitive erythroblasts and some myeloid cells serving the needs of the embryo before mature haematopoietic cells are produced by definitive HSCs (reviewed by Medvinsky *et al.*, 2011).

Definitive haematopoiesis is the second and the last wave of haematopoiesis emerging during embryogenesis and persisting in the adult organism. Although a growing body of data suggests that definitive haematopoiesis originates in the AGM region, this is still a debatable issue (reviewed by Medvinsky *et al.*, 2011).

1.3. Haematopoietic Hierarchy

1.3.1. Adult Haematopoietic Hierarchy

The majority of cells forming the adult haematopoietic system are mature haematopoietic cells including megakaryocytes and platelets, erythrocytes, neutrophil, eosinophil and basophil granulocytes, monocytes and macrophages, T, B and natural killer (NK) cells and myeloid and plasmacytoid dendritic cells. All these cells are derived from HSCs through multistep differentiation (Figure 1.3.1). Although this process is a continuum, different types of haematopoietic progenitors can be distinguished during the transition from HSCs to mature haematopoietic cells. The maturational relationship between HSCs, immature haematopoietic progenitors, committed haematopoietic progenitors and mature haematopoietic cells can be viewed as a hierarchy (reviewed by Seita and Weissman, 2010).

At the foundation of the definitive haematopoietic hierarchy are definitive HSCs, which in adult vertebrates usually reside in the bone marrow. HSCs in the bone marrow are present at a very low frequency. In the mouse bone marrow, there is one HSC in 10000 nucleated cells (Kumaravelu *et al.*, 2002). Isolating by fluorescence-activated cell sorting (FACS) $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}CD150^{+}CD48^{-}CD34^{-}Flt3^{-}$ cells from the mouse bone marrow, HSCs can be enriched almost to a single cell level (Adolfsson *et al.*, 2001; Kiel *et al.*, 2005; Osawa *et al.*, 1996; Spangrude *et al.*, 1988; Wilson *et al.*, 2008). The precise frequency of HSCs in the human bone marrow is not known since the limiting dilution analysis by direct transplantation of unfractionated cells has not been done. However, recalculation of data obtained with purified cell populations suggests that there is one HSC in 25000–50000 nucleated

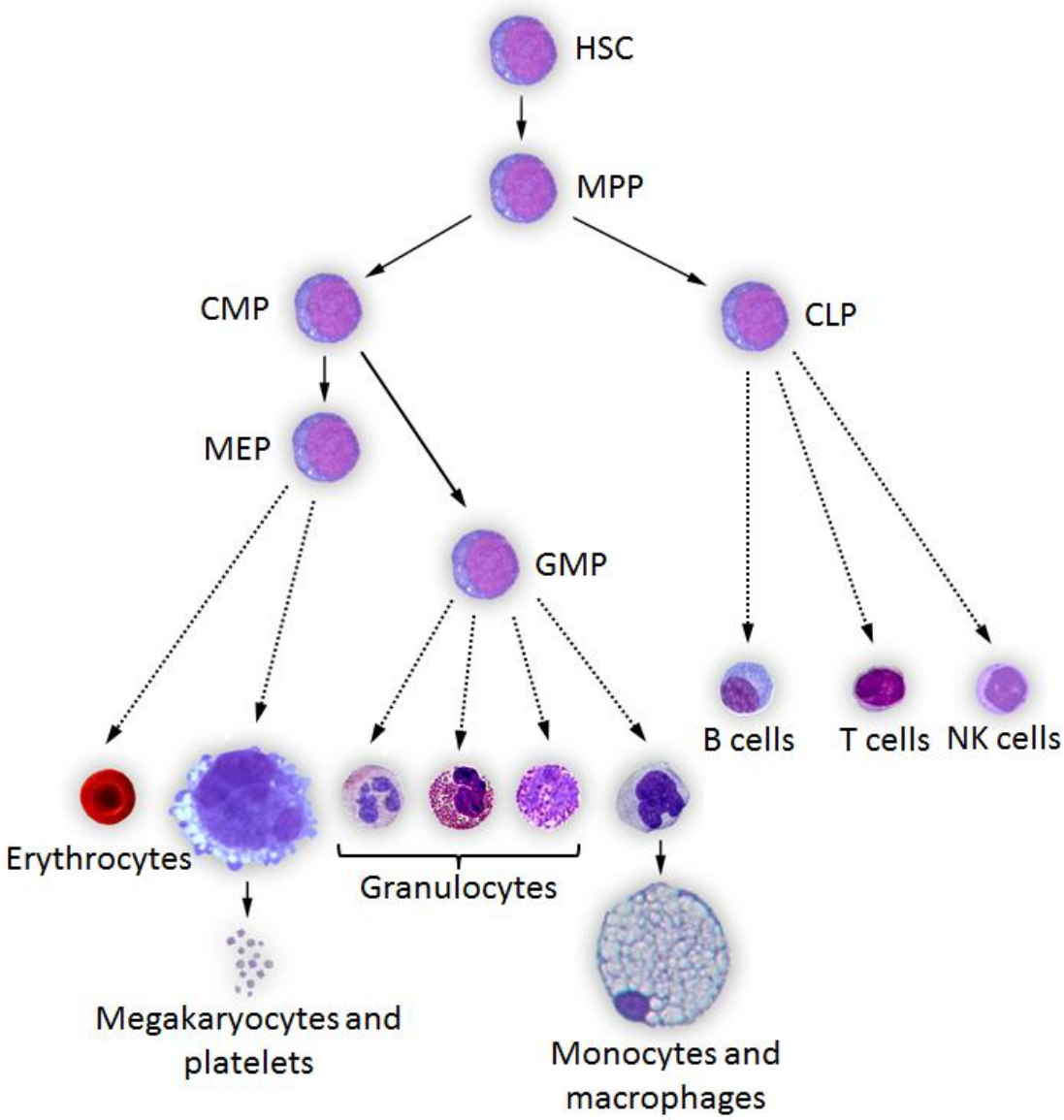


Figure 1.3.1. Adult haematopoietic hierarchy (classical model). The multistep differentiation of definitive HSCs results in the formation of progeny with descending haematopoietic lineage plasticity and self-renewal capacity. Mature haematopoietic cells are terminally differentiated. The dotted lines indicate that intermediate haematopoietic progenitors are not depicted on the diagram. The image was adapted from https://daley.med.harvard.edu/assets/Willy/hematopoiesis.jpg.

human bone marrow cells (Majeti *et al.*, 2007; Notta *et al.*, 2011; Pang *et al.*, 2011). HSCs can be purified from the adult human bone marrow by FACS as $Lin^-CD34^+CD38^-Thy\text{-}1^+CD45RA^-CD49f^+$ cells with frequency 1:10. (Notta *et al.*, 2011). Interestingly, in contrast to mouse HSCs, human HSCs express CD34 and Flt3 (Doulatov *et al.*, 2010).

Immediately downstream of HSCs in the haematopoietic hierarchy, multipotent progenitors (MPPs) have been identified. Similar to HSCs, these cells possess full multilineage differentiation potential; however, they lack unlimited self-renewal capacity (reviewed by Seita and Weissman, 2010). The mouse MPP population is heterogeneous and contains cells with different immunophenotypes listed below in a presumable hierarchical order: $Lin^-Sca\text{-}1^+c\text{-}Kit^+CD150^+CD48^-CD34^+Flt3^-$, $Lin^-Sca\text{-}1^+c\text{-}Kit^+CD150^+CD48^+CD34^+Flt3^-$, $Lin^-Sca\text{-}1^+c\text{-}Kit^+CD150^-CD48^+CD34^+Flt3^-$ and $Lin^-Sca\text{-}1^+c\text{-}Kit^+CD150^-CD48^+CD34^+Flt3^+$ (Adolfsson *et al.*, 2001; Boyer *et al.*, 2011; Christensen and Weissman, 2001; Forsberg *et al.*, 2006; Wilson *et al.*, 2008). Human MPPs are $Lin^-CD34^+CD38^-Thy\text{-}1^-CD45RA^-Flt3^+CD7^-CD10^-$ (Doulatov *et al.*, 2010; Majeti *et al.*, 2007).

Further downstream of MPPs in the haematopoietic hierarchy, common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs) have been identified. Similar to MPPs, these cells possess limited self-renewal capacity. Their differentiation potential is restricted either to the myeloid or lymphoid lineage, respectively. CMPs give rise to megakaryocyte and erythrocyte progenitors (MEPs) and granulocyte and monocyte progenitors (GMPs), which generate platelets and

erythrocytes and granulocytes, monocytes and myeloid dendritic cells, respectively. CLPs give rise to lymphoid lineage-restricted progenitors finally producing T, B, NK and plasmacytoid dendritic cells (reviewed by Seita and Weissman, 2010). By immunophenotype, mouse CMPs, MEPs and GMPs are $Lin^-c\text{-}Kit^+Sca\text{-}1^-CD34^+Fc\gamma RII/III^{lo}$, $Lin^-c\text{-}Kit^+Sca\text{-}1^-CD34^-Fc\gamma RII/III^-$ and $Lin^-c\text{-}Kit^+Sca\text{-}1^-CD34^+Fc\gamma RII/III^{hi}$, respectively (Akashi *et al.*, 2000). Mouse CLPs are $Lin^-Flt3^+IL\text{-}7R\alpha^+$ (Karsunky *et al.*, 2008; Kondo *et al.*, 1997). Human CMPs, MEPs and GMPs are $Lin^-CD34^+CD38^+IL\text{-}3R\alpha^{lo}CD45RA^-$, $Lin^-CD34^+CD38^+IL\text{-}3R\alpha^-CD45RA^-$ and $Lin^-CD34^+CD38^+IL\text{-}3R\alpha^{lo}CD45RA^+$, respectively (Manz *et al.*, 2002). Human CLPs are $Lin^-CD34^+CD38^+Thy\text{-}1^-CD45RA^+CD10^+$ (Galy *et al.*, 1995). Recently, it has been proposed that CLPs do not exist in the human adult haematopoietic hierarchy (Doulatov *et al.*, 2010). Instead, $Lin^-CD34^+CD38^-Thy\text{-}1^{-/lo}CD45RA^+Flt3^+CD10^+$ multilymphoid progenitors (MLPs) have been identified. In contrast to CLPs, MLPs are not lymphoid-lineage restricted and give rise not only to all mature lymphoid cells but also to monocytes and myeloid dendritic cells. As for $Lin^-CD34^+CD38^+Thy\text{-}1^-CD45RA^+CD10^+$ cells, which previously were considered as human CLPs, they lacked T cell differentiation potential and could generate *in vitro* only B and NK cells (Doulatov *et al.*, 2010). Studies in the mouse have also suggested that adult haematopoietic hierarchy may differ from the model described above (Adolfsson *et al.*, 2005; Lai and Kondo, 2006; Mansson *et al.*, 2007).

1.3.2. Embryonic Haematopoietic Hierarchy

In the developing mouse embryo, the haematopoietic hierarchy evolves in the reverse sequence compared to that of the adult haematopoietic hierarchy. The first fully

mature haematopoietic cells, primitive erythroblasts, are found in the mouse yolk sac at day 8–8.5 of development (Silver and Palis, 1997). Around the same time, the first mouse committed haematopoietic progenitors detected *in vitro* as colony-forming units-culture (CFU-Cs) emerge in the yolk sac (Moore and Metcalf, 1970). The first immature haematopoietic progenitors detected *in vivo* as colony-forming units-spleen (CFU-Ss) appear in the mouse embryo around day 9.5–10 (Medvinsky *et al.*, 1993), and the first definitive HSCs are detected in the mouse AGM region approximatelly at day 11.5 of development (Muller *et al.*, 1994).

To explain the emergence of mature haematopoietic cells and haematopoietic progenitors before HSCs, two models have been proposed. The first model suggests that two haematopoietic hierarchies, primitive and definitive, coexist during embryogenesis. The primitive transitory haematopoietic hierarchy emerges early in embryogenesis in the yolk sac and exists temporally until the permanent definitive haematopoietic hierarchy is established by AGM region-derived definitive HSCs. The second model suggests that a single haematopoietic hierarchy persists in the embryo. In this model, some embryonic haematopoietic ancestor cells give rise to the definitive HSC lineage. As HSC specification requires time, the same haematopoietic ancestor cells give rise to haematopoietic progenitors and mature haematopoietic cells to assure the ongoing development of the embryo. When definitive HSCs emerge, the haematopoietic hierarchy finally becomes established. If this model is correct, it cannot be ruled out that the above mentioned haematopoietic ancestor cells emerge in the yolk sac, but that final HSC maturation requires the AGM region environment (reviewed by Medvinsky *et al.*, 2011).

1.4. Assessment of Haematopoietic Potential

As discussed above, the haematopoietic cell population represents a diverse pool of cells that includes HSCs, immature haematopoietic progenitors, committed haematopoietic progenitors and mature haematopoietic cells. To evaluate this diverse population, different assays are used. Assays can be classified according to the cell type that they measure (HSCs, immature haematopoietic progenitors or committed haematopoietic progenitors) and the experimental conditions utilised (*in vivo* or *in vitro*, short-term or long-term). In this section, the main methods used to assess cell haematopoietic potential are described.

1.4.1. Assessment of HSCs

Qualitative (long-term self-renewal and multipotency) and quantitative assessment of HSCs can be performed only *in vivo* using long-term repopulation assays, which can be divided into two major groups: primary and secondary. These can further be categorised as competitive and noncompetitive. Human HSCs are assayed in xenotransplantation models (reviewed by Herbert *et al.*, 2008).

The primary long-term repopulation assay is accepted as the gold standard and the only tool to qualify and quantify HSCs within a transplanted cell population. The competitive assay measures the long-term ability of tested cells to engraft the bone marrow of irradiated recipients, relative to cotransplanted competitor HSCs, which are usually obtained from the bone marrow of a congenic donor with a phenotype distinguishable from the recipient and the tested donor (Szilvassy *et al.*, 1990). The cotransplanted bone marrow cells have a dual role. Firstly, bone marrow

haematopoietic progenitors provide short-term radioprotection to the recipient before long-term HSC engraftment. Secondly, bone marrow HSCs are necessary for semiquantitative assessment of tested HSCs. Homing ability is often considered as an additional HSC defining criterion together with unlimited self-renewal and multilineage engraftment (reviewed by Ema and Nakauchi, 2004; reviewed by Harrison *et al.*, 1993).

The noncompetitive transplantation assay involves transplantation of tested HSCs without competitor cells. This assay more closely reflects allogeneic and autologous transplantation performed in clinical haematology. To provide recipient survival shortly after irradiation, a large number of HSCs need to be transplanted. Alternatively, recipients may be irradiated with a lower dose to ensure the regeneration of their own haematopoietic system. The assay allows evaluation of the myeloablation protection ability of HSCs (reviewed by Herbert *et al.*, 2008).

The secondary repopulation assay is usually performed six months after primary transplantation. To this end, primary recipient bone marrow cells are transplanted into a congenic irradiated secondary recipient that are phenotypically distinguishable from the primary donor. After 4–6 months, the presence of primary donor HSC-derived haematopoietic cells in the secondary recipient is assessed. This gives some indication about HSC long-term self-renewal (reviewed by Herbert *et al.*, 2008).

The xenotransplantation assay is currently the most adequate method to study human HSCs. The first studies were performed using severe combined immunodeficiency

(SCID) recipient mice, which display T and B cell defects (McCune *et al.*, 1988). As SCID mice often mediate the immunorejection of xenograft, they were backcrossed to nonobese diabetic (NOD) mice, which are deficient for NK and antigen-presenting cells (Shultz *et al.*, 1995). Currently, NOD.CB17-*Prkdc*^{scid} (NOD/SCID) mice are the most widely used recipients to study human haematopoiesis *in vivo*. However, a low proportion of human HSCs engraft these animals. In addition, in these mice there is a bias towards B cell differentiation, and the development of T cells is rare. Also, the animals have a short life span (reviewed by Kondo *et al.*, 2003). To address these limitations, NOD/LtSz-*Prkdc*^{scid} *B2m*^{tm1Unc} mice (Kollet *et al.*, 2000) and NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} (NSG) mice (Shultz *et al.*, 2005) were created. The former strain supports a relatively high level of human HSC engraftment but, due to thymic lymphomagenesis, has a short life span. The latter strain supports a higher level of human HSC engraftment and is long-lived. Therefore, NSG mice are the best available xenogeneic recipients able to engraft human HSCs. It is accepted that these mice are considered as engrafted with human HSCs if at least 0.1% of total bone marrow CD45^{+} cells are of human origin (Notta *et al.*, 2010). This criterion was selected to assess the engraftment of human bone marrow, mobilised peripheral blood and umbilical cord blood-derived HSCs.

1.4.2. *In Vivo* Assessment of Immature Haematopoietic Progenitors

To assess immature haematopoietic progenitors *in vivo*, the CFU-S assay is utilised (Till and McCulloch, 1961). Briefly, lethally irradiated mice are transplanted with a tested sample. After seven to 13 days, their spleens are dissected and fixed, and macroscopic spleen colonies are counted. This assay detects the cells that are capable

of short-term self-renewal and myelo-erythroid differentiation. In the definitive haematopoietic hierarchy, CFU-Ss reside downstream of HSCs. CFU-Ss can be divided into early (e.g. day 7) and late (e.g. day 13) CFU-Ss. Spleen colonies generated 7 days after transplantation contain mainly megakaryocytes and erythrocytes. Spleen colonies generated 13 days after transplantation are usually composed of granulocytes, macrophages, megakaryocytes and erythrocytes. Therefore, the late spleen colonies are generated by more immature haematopoietic precursors than the early ones. It is assumed that the number of CFU-Ss is a rough reflection of HSC numbers in a tested cell fraction (reviewed by Herbert *et al.*, 2008). However, pre-CFU-Ss (noncycling bone marrow-repopulating cells that survive cytostatic treatment) were shown to be a better indicator of the number of HSCs in the cell population being transplanted (Hodgson and Bradley, 1979).

1.4.3. *In Vitro* Assessment of Immature Haematopoietic Progenitors

There are two main *in vitro* assays to detect immature haematopoietic progenitors: the long-term culture-initiating cell (LTC-IC) assay and the cobblestone area-forming cell (CAFC) assay.

The LTC-IC assay is a submersed culture system that uses feeder cells to provide substrate and growth factors for the propagation and maturation of primitive haematopoietic progenitors. The LTC-IC is defined as a cell that is able to produce daughter cells that are detectable as colony-forming units-culture CFU-Cs (Dexter *et al.*, 1977). Although LTC-ICs represent a subset of cells that share phenotypic and functional properties with HSCs, these cell types are distinct. Indeed, while human

LTC-ICs are found in both $CD34^+CD38^-$ and $CD34^+CD38^+$ populations; true HSCs are $CD34^+CD38^-$ (Bhatia *et al.*, 1997).

The CAFC assay is also based on a submersed culture system that involves the use of feeder cells. In this assay, immature haematopoietic progenitors migrate underneath the feeder cell layer and at specific time points (defined by their primitiveness) begin to proliferate and form phase-dark haematopoietic colonies or the so-called 'cobblestone areas'. A cell that gives rise to one cobblestone area is called the CAFC. In the mouse CAFC assay, the frequency of day 10 CAFCs correlates with the frequency of day 12 CFU-Ss, and the frequency of day 28 CAFCs correlates with the frequency of HSCs within a tested cell population (Ploemacher *et al.*, 1991). In the human CAFC assay, the frequency of week 6–12 CAFCs correlates with the frequency of HSCs (van Hennik *et al.*, 1998).

1.4.4. Assessment of Committed Haematopoietic Progenitors

The late stages of haematopoiesis correspond to the persistence of committed haematopoietic progenitors that are an intermediate stage between immature haematopoietic progenitors and haematopoietic cells with morphologically visible features of differentiation. Generally, committed myelo-erythroid progenitors are assessed in short-term semisolid medium culture and are called CFU-Cs. When grown in these conditions, they form colonies of different types according to their maturity. The following committed haematopoietic progenitors are usually assessed: colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM), burst-forming unit-erythroid (BFU-E), colony-forming unit-granulocyte,

macrophage (CFU-GM), colony-forming unit-granulocyte (CFU-G) and colony-forming unit-macrophage (CFU-M) (Nissen-Druey *et al.*, 2005).

1.5. Embryonic Development of Haematopoiesis in the Mouse

There are several intra- and extra-embryonic sites of haematopoiesis in the mouse embryo (Figure 1.5.1). In accordance with current views, these sites are the yolk sac, the AGM region, the placenta, the umbilical and vitelline arteries and the liver. Although the mouse is the best characterised *in vivo* model in experimental haematology, the precise site where definitive HSCs emerge in the mouse embryo is still a debatable issue as the first mouse definitive HSCs can be detected at approximately the same time in different intra- and extra-embryonic tissues (de Bruijn *et al.*, 2000; Gekas *et al.*, 2005; Kumaravelu *et al.*, 2002; Muller *et al.*, 1994; Ottersbach and Dzierzak, 2005). This may be explained either by HSC emergence in different tissues or by HSC migration between different sites of the embryo. As mouse definitive HSC lineage specification involves pre-HSC maturation (Rybtsov *et al.*, 2011; Taoudi *et al.*, 2008), possible migration of these cells may complicate the analysis (reviewed by Medvinsky *et al.*, 2011).

1.5.1. Role of the Yolk Sac in Mouse HSC Development

In the mouse embryo, the first sign of haematopoietic differentiation, βH1-globin gene expression, is detected in the yolk sac mesoderm around day 7.5 of development. By day 8, proliferating and differentiating extra-embryonic mesoderm cells form blood islands consisting of primitive haematopoietic cells surrounded by endothelial cells. Shortly after that, the first fully mature haematopoietic cells,

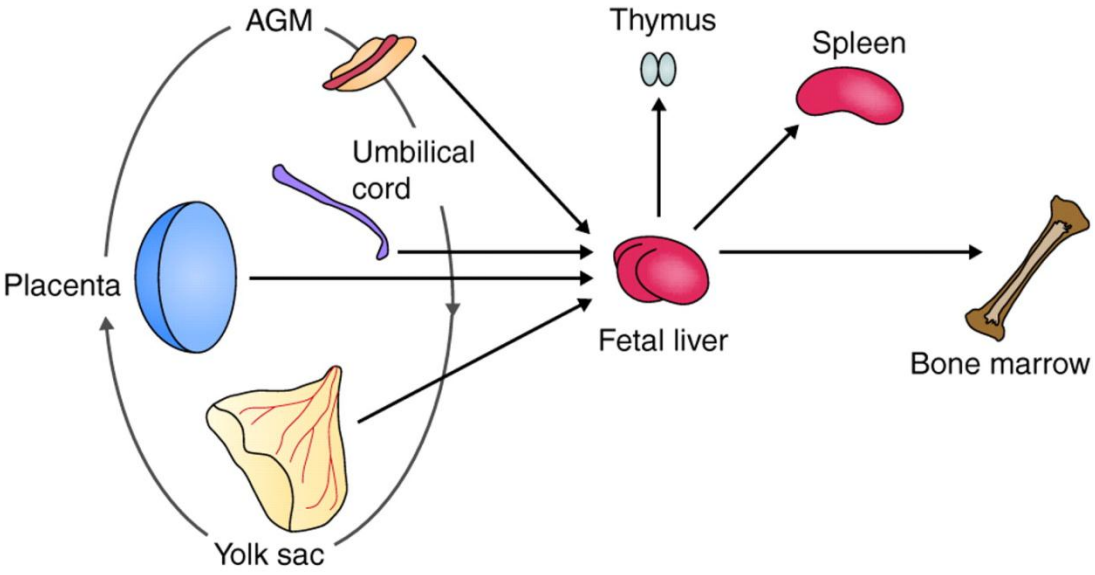


Figure 1.5.1. Multisite development of the haematopoietic system in the mouse embryo. In the mouse embryo, the first definitive HSCs are detected at day 11–11.5 of development in the dorsal aorta, the yolk sac, the umbilical and vitelline arteries and the placenta. Then, HSCs from the above intra- and extra-embryonic sites are believed to migrate to the embryonic liver (day 12.5 of development) and finally to the bone marrow (day 16.5 of development). In meantime, HSCs and/or haematopoietic progenitors colonise the thymus and spleen. The image was taken from Medvinsky *et al.*, 2011.

primitive erythroblasts, are detected in the yolk sac (Silver and Palis, 1997). In 1970, it was reported that the mouse yolk sac harbours significant CFU-C, CFU-S and HSC activity (Moore and Metcalf, 1970). Considering this and the fact that the yolk sac is the earliest site of haematopoiesis in the mouse embryo, it was reasonable to suggest that mouse definitive HSCs emerge in the yolk sac. In addition, earlier observations in the chicken embryo supported this idea (Moore and Owen, 1965; Moore and Owen, 1967).

A few years later, experiments performed with chimeric quail and chicken embryos provided solid evidence against the origin of definitive haematopoiesis in the yolk sac and suggested that definitive HSCs emerge in the embryo proper (Dieterlen-Lievre, 1975). Shortly after that, it was reported that yolk sac cells derived from the day 8 mouse embryo contribute to adult haematopoiesis upon intrauterine transplantation into embryonic recipients (Weissman *et al.*, 1978). Dieterlen-Lievre's finding was interpreted as a peculiarity of the avian but not the mammalian embryo. However, the results of Irving Weissman's experiments have not been independently confirmed.

The ability of the mouse yolk sac to give rise to definitive HSCs was repeatedly questioned in 1990s. Employing a more stringent recipient mouse irradiation protocol to prevent the regeneration of endogenous CFU-Ss, it has been shown that the mouse yolk sac lacks CFU-Ss prior to day 9.5 of development and, of more importance, that the AGM region contains a higher number of CFU-Ss than the yolk sac (Medvinsky *et al.*, 1993). Subsequent studies, have shown that the AGM region

but not the yolk sac is capable of definitive HSC generation through the maturation of pre-HSCs starting from day 10.5 of development and that the first definitive HSCs are detected in the yolk sac only at day 11.5 of development (Medvinsky and Dzierzak, 1996; Muller *et al.*, 1994).

The primary role of the yolk sac in mouse definitive haematopoiesis was suggested again when c-Kit^{+}CD34^{+} yolk sac cells from the day 9 mouse embryo were shown to contribute to adult haematopoiesis upon transplantation into pre-conditioned neonatal recipient mice (Yoder *et al.*, 1997). However, at day 9 of development the circulation in the mouse embryo has already been established, it is unclear whether these cells originate in the yolk sac or in the para-aortic splanchnopleura. To answer this question, definitive HSCs precursors need to be assesses functionally in the early day 8 mouse embryo, when the heart is not yet contracting. As this is not currently possible, only CFU-C migration through the embryonic circulation has been assessed so far (Lux *et al.*, 2008). For this, *Ncx1*$^{-/-}$ mouse embryos, which fail to initiate cardiac contractions and therefore the circulation, were used. It has been reported that the vast majority of CFU-Cs in *Ncx1*$^{-/-}$ mouse embryos reside in the yolk sac, suggesting that intra-embryonic CFU-Cs found in wild-type embryos migrate from the yolk sac. The assessment of HSCs was not possible as the mutant embryos die *in utero* at day 10.5 of development, a day before the first mouse definitive HSCs can be detected. An attempt to show that the HSC lineage emerges in the yolk sac has been made employing genetic labelling of *Runx1*-expressing cells (Samokhvalov *et al.*, 2007; Tanaka *et al.*, 2012). However, considering the unclear duration of labelling, it is difficult to draw firm conclusions from these studies.

The lack of intra-embryonic CFU-Cs in *Ncx1*$^{-/-}$ mouse embryos may be a secondary effect related to the lack of physical stress created by blood flow. It has been shown that shear stress applied *in vitro* improves both haematopoietic differentiation of mouse embryonic stem cells and propagation of mouse para-aortic splanchnopleura and AGM region-derived CFU-Cs *in vitro*. Additionally, intra-embryonic CFU-Cs in *Ncx1*$^{-/-}$ mouse embryos can be rescued by applying shear stress during cell culture (Adamo *et al.*, 2009). The pro-haematogenic effect of blood flow shear stress was also confirmed in the zebrafish embryo (North *et al.*, 2009). As for a mechanistic insight into this phenomenon, the induction of nitric oxide signalling has been suggested as a molecular pathway to stimulate intra-embryonic haematopoiesis in both the mouse and zebrafish embryo (Adamo *et al.*, 2009; North *et al.*, 2009).

Some observations have suggested that there is no controversy as to whether mouse definitive HSC lineage emerges in the yolk sac or the para-aortic splanchnopleura since both tissues are capable of generating definitive HSCs autonomously (Matsuoka *et al.*, 2001). To this end, the yolk sac and the para-aortic splanchnopleura from the day 8 mouse embryo were individually cocultured for 4 days with AGM-S3 stromal cells. This resulted in the production of definitive HSCs both by the yolk sac and by the para-aortic splanchnopleura, suggesting that definitive HSC precursors were present in both tissues prior to the onset of circulation. Despite the exceptional potential of AGM-S3 cells to induce definitive haematopoiesis in the precirculation mouse embryo, no further studies employing this cell line have been published since 2001. This urges us to interpret the above results with care.

1.5.2. Role of the AGM Region in Mouse HSC Development

Although experiments in the avian embryo had already suggested an intra-embryonic origin of definitive HSCs in 1975 (Dieterlen-Lievre, 1975), the view that mammalian HSCs originate in the yolk sac dominated the field until the early 1990s. In 1993, a novel intra-embryonic source of haematopoietic activity, the AGM region, was described. It was reported that at day 9.5 of development the AGM region is the only source of CFU-S activity in the mouse embryo. During day 10, CFU-Ss appear in the yolk sac, with approximately fourfold lower frequency than in the AGM region. The liver does not develop CFU-S activity until day 11–11.5 of development (Medvinsky *et al.*, 1993).

Although the AGM region contains more CFU-S than the yolk sac, it does not necessarily mean that mouse definitive HSCs emerge in the AGM region. When the long-term repopulation assay was employed to assess the spatio-temporal distribution of HSCs in the mouse embryo, it was found that the first definitive HSCs can be easily detected at day 11–11.5 of development both in the AGM region and in the yolk sac. In both sites, only one HSC per tissue was found (Kumaravelu *et al.*, 2002; Muller *et al.*, 1994). It is worth mentioning that definitive HSCs were occasionally detected in the day 10.5 mouse AGM region and never in the day 10.5 yolk sac. To repopulate three irradiated adult recipient mice, 112 day 10.5 AGM regions were required (Muller *et al.*, 1994). This finding was used to favour the AGM region as a primary source of definitive HSCs. However, this did not look like a very convincing argument due to the infrequency of the observation, which more likely could be explained with asynchronous development of some mouse embryos. Since HSCs

were detected in both the AGM region and the yolk sac at approximately the same time of development, it remained unclear in which embryonic site definitive HSCs emerge.

To answer this question, an *ex vivo* organ explant culture system was developed (Medvinsky and Dzierzak, 1996). At day 10.5 of development, definitive HSCs are extremely rare in the mouse embryo (Muller *et al.*, 1994). Dissecting and individually culturing the AGM region, the yolk sac and the liver from the day 10.5 mouse embryo, it has been shown that only cultured AGM region cells are capable of providing robust long-term multilineage haematopoietic repopulation in irradiated adult recipient mice, suggesting that the AGM region but not the yolk sac generates the first mouse definitive HSCs (Medvinsky and Dzierzak, 1996).

The same conclusion was drawn using an *in vitro* myelo-lymphoid differentiation assay. The para-aortic splanchnopleura and the yolk sac from day 7.5–8.5 mouse embryos were individually cocultured with mouse bone marrow-derived S17 stromal cells in the presence of cytokines. After 5 days, cells were transferred either onto fresh S17 stromal cells and cultured for 10–15 days to assess myeloid, B and NK cell potential, or cocultured with mouse thymic lobes for 10–15 days to assess T cell potential. It has been found that the para-aortic splanchnopleura possesses both myeloid and lymphoid differentiation potential prior to the onset of circulation, while the yolk sac produced only myeloid cells. Although the analysis was not performed at a clonal level, it has been suggested that cells with multilineage differentiation

potential, presumably definitive HSCs, emerge in the para-aortic splanchnopleura (Cumano *et al.*, 1996).

After it had been discovered that the mouse AGM region is capable of autonomous HSC generation (Medvinsky and Dzierzak, 1996), further steps were taken to localise the source of HSC activity within the AGM region. Various vertebrate embryos contain endothelium-associated haematopoietic cell clusters attached mainly to the ventral wall of the dorsal aorta (Dantschakoff, 1909; Emmel, 1916; Garcia-Porrero *et al.*, 1995; Jaffredo *et al.*, 1998; Jordan, 1917; Minot, 1912; Tavian *et al.*, 1996). Also, it has been shown that *Runx1*, a key transcription factor essential for definitive HSC embryonic development (reviewed by Swiers *et al.*, 2010), is expressed in the haematopoietic cell clusters and some endothelial cells lining the ventral wall of the dorsal aorta (North *et al.*, 1999). The above observations indicated that the dorsal aorta might be a source of HSC activity within the mouse AGM region. Subdissecting the day 11.5 mouse AGM region into the dorsal aorta and the UGRs and individually transplanting cell suspensions prepared from these tissues into irradiated adult recipient mice, it has been found that only the dorsal aorta harbours HSCs (de Bruijn *et al.*, 2000). Subdissecting the day 11.5 mouse dorsal aorta and surrounding mesenchyme along the midline into the ventral domain (AoV) and the dorsal domain (AoD) and testing these two tissues for HSC activity in the long-term repopulation assay, it has been found that the vast majority of definitive HSCs reside in the AoV. Moreover, it has been shown that exclusively the AoV is capable of HSC generation and expansion in the organ explant culture system (Taoudi and Medvinsky, 2007).

As it has already been mentioned, at day 11.5 of development the mouse AGM region contains one definitive HSC (Kumaravelu *et al.*, 2002; Muller *et al.*, 1994). After 3–5 days of culture in the organ explant culture system, the number of definitive HSCs increases up to 12 (Kumaravelu *et al.*, 2002). A striking 150-fold increase of HSC numbers occurs in the reaggregate culture system after 4 days of culture in the presence of cytokines. The method involves the dissociation of the AGM region, purification of the cell population of interest, reaggregation of this population with primary AGM region stromal cells and culture at the gas-liquid interface (Sheridan *et al.*, 2009; Taoudi *et al.*, 2008). Using the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE), it has been shown that the most of HSC within the reaggregated AGM region cultured for 4 days underwent no more than four divisions. In order to generate 150 HSCs from a single HSCs present in the mouse AGM region before culture, eight symmetrical divisions would be required. Therefore, the generation of mouse definitive HSCs *in vitro* and presumably *in vivo* occurs through the maturation of HSC ancestors termed pre-HSCs (Taoudi *et al.*, 2008), the existence of which was already suspected by Medvinsky and Dzierzak (1996).

The mouse pre-HSCs described by Taoudi *et al.* (2008) are now called type II pre-HSCs in order to distinguish them from type I pre-HSCs, which were described later (Rybtsov *et al.*, 2011). Mouse type II pre-HSCs share the same phenotype with definitive HSCs and are $CD144^{+}CD45^{+}$ (Taoudi *et al.*, 2008; Taoudi *et al.*, 2005). Developing a new coaggregate culture system in which the population of interest is coagregated with mouse OP9 stromal cells instead of primary AGM region stromal

cells, previously undetectable $CD144^{+}CD45^{-}CD41^{+}$ pre-HSCs have been discovered. These pre-HSCs were termed type I pre-HSCs because they mature into definitive mouse HSCs via the intermediate $CD144^{+}CD45^{+}$ type II pre-HSC phenotype (Rybtsov *et al.*, 2011).

Considering that mouse definitive HSCs, unlike pre-HSCs, are capable of long-term high-level haematopoietic repopulation upon direct transplantation into irradiated adult wild-type (immunocompetent) recipient mice, type I and type II pre-HSCs (Rybtsov *et al.*, 2011; Taoudi *et al.*, 2008) may not be the only pre-HSCs described to date. For example, $c\text{-}Kit^{+}CD34^{+}CD41^{+}$ long-term repopulating haematopoietic cells found in the yolk sac and the para-aortic splanchnopleura of the day 9 mouse embryo can repopulate preconditioned neonatal but not adult recipient mice (Ferkowicz *et al.*, 2003; Yoder *et al.*, 1997). This suggests that these cells are not yet fully developed HSCs, and to become definitive HSCs they require some undetermined environmental factors present in the neonatal organism. As another example, $c\text{-}Kit^{+}AA4.1^{+}CD31^{+}CD41^{+}CD45^{-/lo}$ long-term repopulating haematopoietic cells found in the intra-aortic cell clusters and subaortic patches in the day 10.5 mouse embryo can be mentioned. Importantly, these cells repopulate irradiated immunodeficient but not wild-type adult recipient mice (Bertrand *et al.*, 2005). This suggests that they are not definitive HSCs since to achieve successful haematopoietic engraftment they lack mechanisms to resist the immune system of wild-type hosts. Moreover, even in immunodeficient recipients, these cells are not capable of high-level haematopoietic repopulation (Bertrand *et al.*, 2005). Therefore, the described

above long-term repopulating haematopoietic cells may be pre-HSCs; however, the link between them and type I and type II pre-HSCs remains to be established.

1.5.3. Role of Extra-Embryonic Arteries in Mouse HSC Development

It has been found that during days 11.5 and 12.5 of development the mouse umbilical and vitelline arteries contain some numbers of definitive HSCs. The number of HSCs in these blood vessels was estimated as one HSC per two combined arteries, which is comparable to HSC frequency in the day 11.5 AGM region (de Bruijn *et al.*, 2000). The first HSCs could be detected in the umbilical and vitelline arteries already at day 10.5 of development, but HSC frequency is as low as in the day 10.5 mouse AGM region (de Bruijn *et al.*, 2000; Muller *et al.*, 1994). Although definitive HSCs are detected in the umbilical and vitelline arteries, these vessels failed to generate HSC in the organ explant culture system (de Bruijn *et al.*, 2000). Thus, the ability of these vessels to initiate definitive haematopoiesis autonomously remains unclear.

Recently, it has been suggested that the vitelline artery (also termed the omphalomesenteric artery) plays a role in the establishment of mouse definitive haematopoiesis through extravascular emergence of haematopoietic cell clusters and presumably definitive HSCs (Zovein *et al.*, 2010). At day 9 of development, the vitelline artery is directly connected to the umbilical arteries. This connection is lost by day 11, when the vitelline artery establishes a connection to the dorsal aorta. In order to achieve this, between days 9 and 11 of development the vitelline artery undergoes significant remodelling. At the beginning, the link to the dorsal aorta occurs through a network of multiple small blood vessels. When these blood vessels

undergo remodelling to establish a single connection to the dorsal aorta, large endothelium-associated haematopoietic cell clusters, previously termed mesenteric blood islands, emerge (Garcia-Porrero *et al.*, 1995; Zovein *et al.*, 2010). By analysing this process in two dimensions, Zovein *et al.* (2010) have concluded that the haematopoietic cell clusters containing definitive HSCs emerge through extravasation. It is important to know whether emerging HSCs are located inside or outside blood vessels since this can shed light on the mechanism of HSC migration to the liver. Importantly, the above conclusion regarding the extravasation of haematopoietic cell clusters was declined when three-dimensional whole embryo immunostaining and imaging technique was employed. All endothelium-associated haematopoietic cell clusters observed around the vitelline artery of day 10–10.5 mouse embryos were intra-arterial (Yokomizo *et al.*, 2011).

1.5.4. Role of the Placenta in Mouse HSC Development

When, 35 years ago, long-term chronic anaemia correction was achieved in c-Kit$^{W/Wv}$ mice transplanted with wild-type placental cells, it was suggested for the first time that the mouse placenta harbours HSCs (Dancis *et al.*, 1977). A renewed interest in the placenta as a haematopoietic organ emerged again in 2000s, after two research groups had independently shown that between days 11.5 and 15.5 of development the mouse placenta serves as a reservoir for definitive HSCs (Gekas *et al.*, 2005; Ottersbach and Dzierzak, 2005).

The first HSCs can be detected in the mouse placenta at day 10.5 of development; however, these HSCs are extremely rare and provide only low-level repopulation

(Gekas *et al.*, 2005). The first high-level repopulating HSCs are detected in the mouse placenta at day 11.5 of development (Gekas *et al.*, 2005; Ottersbach and Dzierzak, 2005), approximately at the same time when they are detected in the AGM region, the yolk sac and the umbilical and vitelline arteries (de Bruijn *et al.*, 2000; Kumaravelu *et al.*, 2002; Muller *et al.*, 1994). At day 11.5, there are no more than 2.5 definitive HSCs per one mouse placenta (Gekas *et al.*, 2005). By day 12.5 of development, the number of placental HSCs peaks. The actual number of definitive HSCs in the mouse placenta at day 12.5 is unclear and has been reported to vary from 12 (Ottersbach and Dzierzak, 2005) to 50 (Gekas *et al.*, 2005). At day 13.5, placental HSC numbers begin do decline and reach 2–3 HSCs per one mouse placenta by day 15.5 of development, which may be explained by the relocation of placental HSCs to the liver (Gekas *et al.*, 2005).

Since the circulation in the mouse embryo is established 3 days before the first HSCs can be detected in the placenta, it is unclear whether mouse placental HSCs originate in the placenta, the AGM region or the yolk sac. The mouse placenta, unlike the day 10.5–11.5 AGM region (Kumaravelu *et al.*, 2002; Medvinsky and Dzierzak, 1996) and the day 12.5 yolk sac (Kumaravelu *et al.*, 2002), was unable to produce HSCs in the organ explant culture system (Ottersbach and Dzierzak, 2005; Robin *et al.*, 2006). Therefore, it is possible that either the mouse placenta does not contain pre-HSCs and is colonised by HSCs emerging elsewhere or that due to some technical reasons the explant culture system does not support placental pre-HSCs.

The role of the circulation in the establishment of placental haematopoiesis was assessed *in vivo* employing the same approach as in the case with the yolk sac. It has been shown that in *Ncx1*$^{-/-}$ mouse embryos, which fail to initiate cardiac contractions and therefore the circulation, CD41^{+} haematopoietic cell clusters emerge in the vessels of the chorioallantoic mesenchyme and developing labyrinth. Furthermore, placental cells from the mutant embryos are capable of myelo-erythroid and lymphoid differentiation *in vitro* (Rhodes *et al.*, 2008). All this suggests that the mouse placenta possesses independent haematopoietic activity. However, it was not possible to assess if the mouse placenta can independently generate definitive HSCs as *Ncx1*$^{-/-}$ mouse embryos die *in utero* at day 10.5 of development, before the appearance of transplantable HSCs. Importantly, organ explant culture experiments have suggested that the mouse placenta cannot generate HSCs (Ottersbach and Dzierzak, 2005; Robin *et al.*, 2006). Additionally, it has been reported that definitive HSCs in the mouse placenta are CD41^{-} (Robin *et al.*, 2011). This suggests that the emergence of CD41^{+} cell clusters in the placental vasculature and other signs of independent haematopoietic activity may not be related to the emergence of definitive HSCs.

1.6. Embryonic Development of Haematopoiesis in Birds

The first systematic studies addressing the embryonic origin of HSCs were performed on the chicken embryo. Parabiosis and cell transplantation between chicken embryos of opposite sexes, suggested that the yolk sac is the most likely source of the first HSCs (Moore and Owen, 1965; Moore and Owen, 1967). The first study that has brought this dogma into a question was also performed on avian

embryos (Dieterlen-Lievre, 1975). Grafting 2-day-old quail embryos onto chicken yolk sacs of the comparable developmental stage and employing morphological differences between quail and chicken cell nuclei showed that after 6–11 days of incubation all haematopoietic cells in the chimeric embryos were of quail origin, suggesting that HSCs emerge in some intra-embryonic site but not in the yolk sac. An intracoelomic grafting of quail spleen or thymus rudiments into the chicken embryo confirmed that these organs can be colonised by chicken haematopoietic cells, excluding inter-species incompatibility in the system (Dieterlen-Lievre, 1975).

To find the possible intra-embryonic source of HSCs, the avian embryo was examined at 4–5 days of development, and some basophilic cells were found in the dorsal mesentery. These cells were proposed to be the earliest haematopoietic precursors. After 2–3 days, haematopoietic cells have been found to form dense foci at the level of the cardinal veins infiltrating the wall of blood vessels and lymph spaces and protruding into the lumen of the dorsal aorta. The intra-embryonic origin of the haematopoietic cells was confirmed in the chimeric embryo system (Dieterlen-Lievre and Martin, 1981). The study suggested that HSCs in the avian embryo may originate from underneath the luminal layer of the dorsal aorta. However, later the same research group proposed a revised scenario for the embryonic development of HSCs in birds. By labelling the vasculature of the chicken embryo with DiI-conjugated acetylated low-density lipoproteins at the time when intra-aortic haematopoietic cell clusters were not yet present, it was found that after the emergence of the clusters haematopoietic cells within the clusters retain DiI dye. As at the time of the labelling the aortic endothelium consisted entirely of $VEGFR2^{+}$ flat

endothelial cells with no evidence of $CD45^+$ haematopoietic cells present, it was concluded that haematopoietic cell clusters and presumably HSCs and/or pre-HSCs within these clusters were derived from endothelial cells at day 3–3.5 of development (Jaffredo *et al.*, 1998). Subsequently, the labelled haematopoietic cells form the intra-aortic clusters ingressed in the dorsal mesentery ventral to the dorsal aorta, propagated and formed para-aortic haematopoietic cell foci. This observation was confirmed using cell labelling with nonreplicative *LacZ*-bearing retroviruses (Jaffredo *et al.*, 2000).

Shortly after the emergence of HSCs in the dorsal aorta, the thymus, bursa of Fabricius and bone marrow of the avian embryo are colonised by haematopoietic cells. The colonisation of the thymus occurs during several short periods, with the first between days 5 and 6 in the quail embryo and between days 7 and 8.5 in the chicken embryo. The bursa of Fabricius is colonised by haematopoietic cells during a single period between days 7 and 11 in the quail embryo and days 8 and 14 in the chicken embryo. The bone marrow is colonised by haematopoietic cells on day 9 in the quail embryo and on day 10 in the chicken embryo (reviewed by Dieterlen-Lievre and Le Douarin, 2004).

1.7. Embryonic Development of Haematopoiesis in Lower Vertebrates

Due to external embryonic development, a high embryo tolerance for manipulations, prolific reproduction and comparatively low maintenance cost, frogs and fish are powerful tools in developmental biology. To study haematopoiesis during

embryogenesis, the African clawed frog (*Xenopus laevis*) and the zebrafish (*Danio rerio*) are frequently used (reviewed by Ciau-Uitz *et al.*, 2010; reviewed by Paik and Zon, 2010).

1.7.1. Haematopoiesis in the Frog Embryo

In the frog embryo, primitive haematopoiesis occurs in the ventral blood island (Turpen *et al.*, 1981). Its anterior portion starts producing haematopoietic cells, mainly of myeloid lineage, at 24 h post fertilisation (hpf) (Ohinata *et al.*, 1990). The posterior portion of the ventral blood island gives rise to embryonic erythrocytes, myeloid cells and short-lived T cells at 35 hpf (Maeno *et al.*, 1985).

As for definitive haematopoiesis in the anuran embryo, it originates in the dorso-lateral plate mesoderm giving rise to the trunk portion of the dorsal aorta, where cell clusters containing presumably definitive HSCs are formed at 80–92 hpf (Ciau-Uitz *et al.*, 2000; Maeno *et al.*, 1985; Turpen *et al.*, 1981). Importantly, the ventral blood island and the dorso-lateral plate mesoderm derive from different blastomeres, suggesting that definitive HSCs emerge independently of primitive haematopoietic cells. (Ciau-Uitz *et al.*, 2000). During the larval period, the main haematopoietic organ in the frog becomes the liver populated by HSCs from the dorsal aorta (Chen and Turpen, 1995). The bone marrow takes this function over after metamorphosis (Hadji-Azimi *et al.*, 1987).

1.7.2. Haematopoiesis in the Zebrafish Embryo

Being almost transparent, the zebrafish embryo provides a unique opportunity to study the development of haematopoiesis *in vivo* in real time (Bertrand *et al.*, 2010; Kissa and Herbomel, 2010). There are two sites of primitive haematopoiesis in the zebrafish embryo: the rostral blood island, which develops from the anterio-lateral plate mesoderm and gives rise to myeloid cells, mainly macrophages, starting from 10 hpf (Herbomel *et al.*, 1999), and the intermediate cell mass, which develops from the posterio-lateral plate mesoderm and gives rise to erythroid cells entering the circulation around 24 hpf (Long *et al.*, 1997).

It is believed that definitive HSCs in the zebrafish embryo emerge in the dorsal aorta through the so-called endothelio-haematopoietic transition at 35–36 hpf (Bertrand *et al.*, 2010; Kissa and Herbomel, 2010). The endothelio-haematopoietic transition starts about 30 hpf with aortic floor endothelial cell contractions and bending towards the subaortic space (Figure 1.7.1). After 1–2 h, the bent cells contract through the medio-lateral axis and loose contacts with adjacent lateral cells retaining contacts with rostral and caudal cells. Then, the distal contacts also disrupt and the cells undergoing the endothelio-haematopoietic transition acquire haematopoietic progenitor morphology and enter first the subaortic space, then the axial vein and finally the circulation (Kissa and Herbomel, 2010). It is believed that after emerging in the dorsal aorta definitive HSCs migrate into the caudal haematopoietic tissue and expand there. Starting from the second week of development, HSCs migrate from the caudal haematopoietic tissue into the kidney marrow, which is the main

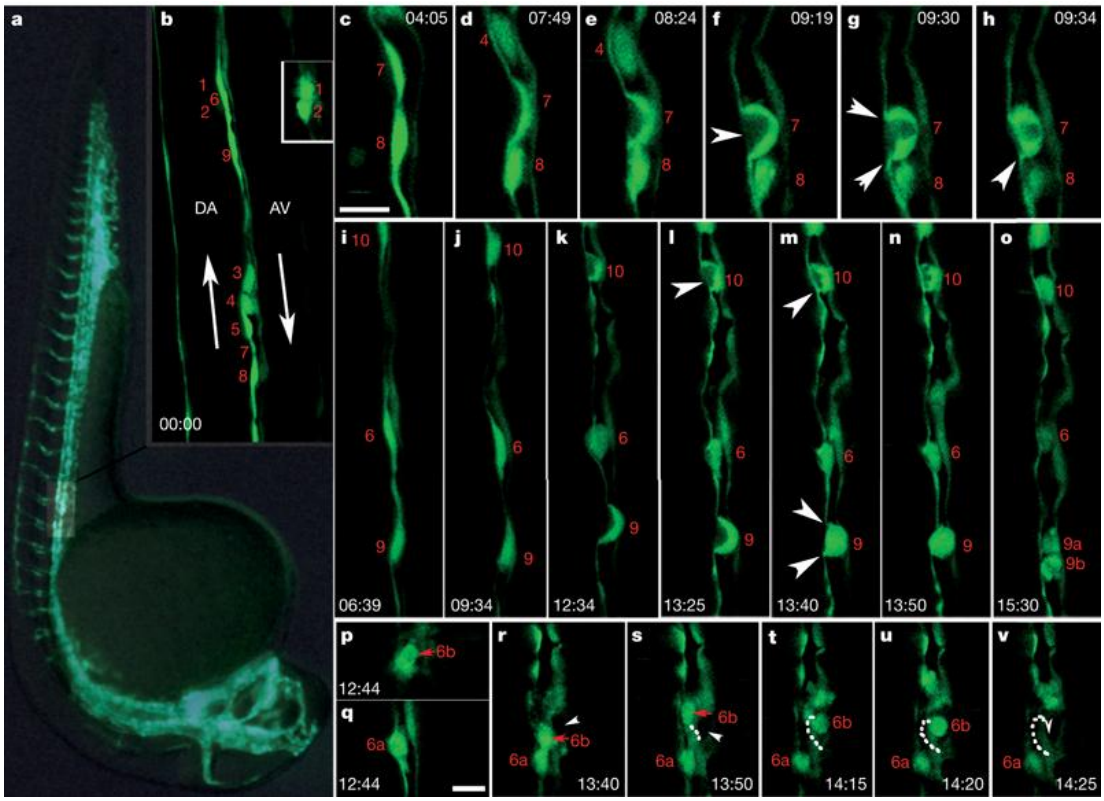


Figure 1.7.1. Endothelio-haematopoietic transition in the zebrafish embryo. GFP fluorescence highlights the whole vasculature in the KDR-GFP zebrafish embryo. The sequential stages of the endothelio-haematopoietic transition are explained in the text. Arrowheads point to haematopoietic precursor cells. DA, dorsal aorta. AV, axial vein. The image was taken from Kissa and Herbomel, 2010.

haematopoietic organ in the late larva and adult zebrafish (Bertrand *et al.*, 2008; Murayama *et al.*, 2006).

1.8. Embryonic Development of Haematopoiesis in the Human

For a long time, it was almost impossible to access the early human embryo for research purposes. Therefore, the investigation on the embryonic development of the human haematopoietic system has not been performed systematically. In the late 1980s, the antiprogestative compound mifepristone became routinely used worldwide to terminate early pregnancies, which allowed obtaining relatively intact early human embryos for research purposes (Vervest and Haspels, 1985). At the same time, the first immunodeficient mice able to accept human HSC grafts were generated (McCune *et al.*, 1988). This enabled the start of studies investigating the embryonic development of the human haematopoietic system and focusing on the origin of definitive HSCs.

Until now, the development of the haematopoietic system in the early human embryos was characterised mainly by immunohistochemical methods and *in vitro* assays (Bloom and Bartelmez, 1940; Huyhn *et al.*, 1995; Luckett, 1978; Migliaccio *et al.*, 1986; Oberlin *et al.*, 2002; Tavian *et al.*, 1996; Tavian *et al.*, 1999; Tavian *et al.*, 2001). Human HSCs have been extensively studied in foetal, neonatal and adult cell sources by transplantation into immunodeficient mice (Conneally *et al.*, 1997; Larochelle *et al.*, 1996; Wang *et al.*, 1997), but at early embryonic stages HSCs were assayed only in the liver (Oberlin *et al.*, 2010) and the placenta (Robin *et al.*, 2009).

Notably, neither the human AGM region nor the yolk sac has ever been assessed for HSC activity.

1.8.1. Intra-Aortic Haematopoietic Cell Clusters

Similar to other vertebrate embryos, the first human haematopoietic cells, primitive erythroid cells, are produced in the yolk sac between days 16 and 18.5 of development (Bloom and Bartelmez, 1940; Luckett, 1978). Classical experiments in the avian model system performed by Dieterlen-Lièvre (1975) and in the mouse model system performed by Medvinsky *et al.* (1993) demonstrated intra-embryonic haematopoietic activity. Bruno Peault and colleagues made the first attempt to extend these observations to the human embryo (Oberlin *et al.*, 2002; Tavian *et al.*, 1996; Tavian *et al.*, 1999; Tavian *et al.*, 2001).

Employing immunohistochemistry, Tavian *et al.* (1996) have shown the existence of temporally and spatially localised $CD34^{+}CD45^{+}$ cell clusters on the ventral wall of the dorsal aorta in the 30- to 37-day-old human embryo. A similar anatomic localisation display endothelium-associated haematopoietic cell aggregates on the ventral wall of the dorsal aorta in the avian and the mouse embryo at equivalent stages of embryonic development (Jaffredo *et al.*, 1998; Taoudi *et al.*, 2008; Yokomizo and Dzierzak, 2010). Although it has never been tested directly, it is assumed that these cell clusters contain HSCs. Tavian *et al.* (1996) assessed the haematogenic potential of the dorsal aorta by its ability to generate CFU-Cs in methylcellulose medium after primary short-term coculture with bone marrow stromal cells. Although in current literature Bruno Peault and colleagues are credited

for the description of human intra-aortic haematopoietic cell clusters, it should be noted that the presence of such clusters on the ventral wall of the human dorsal aorta was reported for the first time in the beginning of the last century (Minot, 1912).

1.8.2. Chronology of the Emergence of Haematopoietic Progenitors

Employing immunohistochemistry and *in vitro* assays, Tavian *et al.* (1999) have described the chronology of the emergence of haematopoietic progenitors in the human embryo. Primitive haematopoietic cells, erythroblasts and some unspecified $CD45^+$ cells, were found in the human yolk sac at day 21 of development (the earliest stage described in the paper). At this embryonic age, there were no $CD45^+$ cells detected in the embryo proper. However, some primitive erythroblasts were found inside the cardiac cavity, suggesting that at day 21 circulation had already been established. The first $CD45^+$ cells were detected inside the human embryo at day 22 of development. The authors have suggested that these cells migrated from the yolk sac.

As for definitive haematopoiesis, it has been established that at day 27 of development the first $CD34^+CD45^+$ cells emerged in the pre-umbilical region of the dorsal aorta. After 3 days, these cells were detected in the vitelline artery and liver. At day 32–33 of development, the number of $CD34^+CD45^+$ cells in the dorsal aorta reaches several hundred. These cells disappeared from the dorsal aorta by day 40. Instead, their number increases significantly in the liver.

The chronology described above is based on the assumption that the earliest haematopoietic progenitors in the human embryo are $CD34^+$. Although the authors demonstrated that the $CD34^+$ cell population possesses haematopoietic activity giving rise to haematopoietic colonies in methycellulose, it was not shown that $CD34^-$ cells in the early human embryo are void of haematopoietic potential (Tavian *et al.*, 1999). If the earliest human embryonic haematopoietic progenitors are indeed $CD34^-$, their spatio-temporal distribution in the human embryo may differ from the described above.

1.8.3. Dorsal Aorta but not the Yolk Sac Contains Multilineage Haematopoietic Progenitors

To assess further the beginning of the development of definitive haematopoiesis in the human embryo, Tavian *et al.* (2001) employed a multistep *in vitro* assay. To this end, the dorsal aorta and the yolk sac were dissected and individually seeded intact on monolayers of confluent mouse bone marrow-derived MS-5 stromal cells and cultured in the presence of cytokines for 3 days. Then, tissues were dissociated and cultured for 10 additional days under the same conditions. Finally, cells were transferred either onto fresh MS-5 stromal cells and cultured for 4–5 weeks to assess myeloid, B and NK cell differentiation potential, or cocultured with mouse thymic lobes for 20 days to assess T cell differentiated potential.

Using this *in vitro* assay, it has been demonstrated that the yolk sac at all stages assessed is capable of generating only myloid and NK cells whereas the dorsal aorta starting from day 24 of development can give rise to myeloid, B, T and NK cells.

The authors proposed that human multipotent haematopoietic progenitors and presumably HSCs emerge in the dorsal aorta (Tavian *et al.*, 2001). Since the analysis was not performed at a clonal level, one cannot be sure that the progenitors described were indeed multipotent but not separate myeloid and lymphoid haematopoietic progenitors. Additionally, considering possible coexistence of the embryonic and adult haematopoietic hierarchies during development, *in vitro* assays cannot inform on the development of true HSCs, which can be assessed only *in vivo* using long-term repopulation assays.

1.8.4. Haematopoietic Potential of the Vascular Endothelium

Employing *in vitro* assay described in the previous section, Oberlin *et al.* (2002) made an attempt to establish the haematogenic potential of the aortic and vitelline endothelium in 28- to 44-day-old human embryos. The authors have found that all vascular endothelial cells in the human embryo express CD34. None of the $CD45^+$ cells were ever found to be positive for CD34 expression before day 28 of development. After day 28, $CD34^+CD45^+$ cell clusters were found on the ventral wall of the dorsal aorta. It was concluded that all vascular endothelial cells in the human embryo express CD34 and can be distinguished from haematopoietic progenitors as only the latter express CD45. The so-called endothelial $CD34^+CD45^-$ cell population purified from the human aorta and the vitelline artery gives rise to myeloid and lymphoid cells. The authors concluded that a subset of human embryonic endothelial cells is haematogenic.

Considering recent findings in other model systems, especially in the zebrafish embryo (Bertrand *et al.*, 2010; Kissa and Herbomel, 2010), it is very likely that the haematogenic endothelium also exists in the human embryo. However, the data obtained by Oberlin *et al.* (2002) are ambiguous in this respect. During human embryonic stem (ES) cell *in vitro* haematopoietic differentiation, CD43 marks emerging haematopoietic progenitors before CD45 (Vodyanik *et al.*, 2006). In the mouse embryo, the earliest haematopoietic progenitors express CD41 but not CD45 (Ferkowicz *et al.*, 2003; Mikkola *et al.*, 2003). The same is true for *in vitro* haematopoietic differentiation of mouse ES cells (Eilken *et al.*, 2009; Lancrin *et al.*, 2009). Therefore, the conclusion regarding the haematogenic potential of the human embryonic endothelium cannot be made without showing that $CD34^{+}CD45^{-}$ cells lack the expression of CD43 or CD41.

1.8.5. Role of the Placenta in Human HSC Development

In the mouse placenta, definitive HSCs are detected at the same developmental age as in the AGM region, the yolk sac and the umbilical and vitelline arteries. Therefore, the placenta is considered to be one of the HSC sources in the mouse embryo (de Bruijn *et al.*, 2000; Gekas *et al.*, 2005; Kumaravelu *et al.*, 2002; Muller *et al.*, 1994; Ottersbach and Dzierzak, 2005). In the human embryo, the placenta also possesses haematopoietic activity. Already at week 5–6 of development (the earliest age studied), the human placenta contains comparatively high numbers of cells with HSC immunophenotype and CFU-Cs, which are enriched within the $CD34^{hi}CD45^{lo}$ cell population (Barcena *et al.*, 2009; Robin *et al.*, 2009). It has also been reported that the placenta harbors HSCs from at least week 6 of development, as was assessed

by highly sensitive PCR analysis (Robin *et al.*, 2009). However, this method is not specific for haematopoietic cells and in principal can detect any donor-derived placental cells that survived after transplantation. The presence of true multipotent HSCs in the placenta was convincingly shown only starting from week 9 of development, when human haematopoietic engraftment in NSG recipient mice was assessed by flow cytometry (Robin *et al.*, 2009). Thus, the exact timing of HSC appearance in the placenta remains to be clarified.

1.9. Cellular Origin of Definitive HSCs

More than a century ago, it has been noted that in the embryo the development of the haematopoietic and endothelial lineages is tightly linked together. The observation that during early embryogenesis proliferating and differentiating yolk sac mesoderm cells form blood islands consisting of primitive haematopoietic cells surrounded by endothelial cells led to an idea that both haematopoietic and endothelial lineages arise from common precursor termed haemangioblasts (Sabin, 1920). Around the same time, endothelium-associated intra-aortic haematopoietic cell clusters have been described, and it has been suggested that definitive HSCs emerge from the haematogenic endothelium (Dantschakoff, 1909; Emmel, 1916; Jordan, 1917; Minot, 1912). During the last century, scientific data supporting the above two and some other scenarios have been presented; however, the cellular origin of definitive HSCs is still not completely understood.

1.9.1. Haemangioblasts as HSC Lineage Precursors

Early observations in the chicken embryo have suggested that yolk sac blood islands are clonal structures emerging from individual haemangioblasts as a result of their haemato-endothelial differentiation (Murray, 1932; Sabin, 1920). Therefore, it was reasonable to hypothesise that definitive HSCs develop in the yolk sac from haemangioblasts (Moore and Metcalf, 1970; Moore and Owen, 1965; Moore and Owen, 1967). Although the clonal origin of blood islands in the chicken yolk sac was suggested by morphological observations (Minko *et al.*, 2003), blood islands in the mouse yolk sac have been reported to be multiclonal (Ueno and Weissman, 2006). In fact, there are no blood islands in the mouse yolk sac itself but a noncompartmentalised belt-like structure that encircles the whole yolk sac (Ferkowicz and Yoder, 2005). Furthermore, it is currently widely accepted that definitive HSCs originate intra-embryonically but not in the yolk sac (Dieterlen-Lievre, 1975; Medvinsky *et al.*, 1993). Therefore, the haemangioblast theory of the origin of definitive HSCs has been transformed to be in line with the concept that definitive HSCs emerge in the embryo proper, and it has been speculated that haemangioblasts reside in the para-aortic splanchnopleura (Huber *et al.*, 2004).

Initially, experiments performed with embryonic tissues have provided limited scientific evidence to support the existence of haemangioblasts. In the chicken embryo, hypothetical haemangioblasts ($VEGFR2^+$ cells sorted from extra-embryonic mesoderm) produced *in vitro* either haematopoietic or endothelial colonies but never mixed haemato-endothelial colonies (Eichmann *et al.*, 1997). Observations in the mouse embryo have not provided clear-cut evidence for the existence of

haemangioblasts either. Orthotopic transplantations have shown that haematopoietic and endothelial cells in the mouse yolk sac originate from different regions of the primitive streak, excluding their origin from a single haemato-endothelial precursor (Kinder *et al.*, 1999). However, more recently very rare Flk-1^{+}Brachyury^{+} progenitors giving rise *in vitro* to haematopoietic, endothelial and smooth muscle cells have been identified mainly in the posterior region of the primitive streak of the mouse embryo. Importantly, these cells were rarely detected in the yolk sac, indicating that the yolk sac is colonised by haematopoietic and endothelial progenitors after the segregation of these lineages. The full differentiation potential of the Flk-1^{+}Brachyury^{+} 'haemangioblasts' needs to be elucidated further (Huber *et al.*, 2004).

An additional *in vivo* proof for the existence of haemangioblasts was obtained in the zebrafish embryo. Employing single cell fate maps obtained upon laser activation of caged fluorescein dextran, it has been demonstrated that haemangioblasts reside in the ventral margin of the gastrulating zebrafish embryo. These cells have been shown to be truly bipotent since they gave rise to haematopoietic and endothelial cells only. However, not all haematopoietic and endothelial cells arise from common progenitors, suggesting that some other mechanisms for the emergence of haematopoietic cells may exist during embryogenesis. It is also not clear whether definitive HSCs arise from haemangioblasts in the zebrafish (Vogeli *et al.*, 2006).

Another line of evidence to support the existence of haemangioblasts comes from studies employing *in vitro* haematopoietic differentiation of mouse ES cells.

Although this system may misrepresent true *in vivo* situation, Flk-1^+ *in vitro* haemangioblasts termed blast colony-forming cells have been identified (Choi *et al.*, 1998; Kennedy *et al.*, 1997). The blast colony-forming cells transiently exist within embryoid bodies, three-dimensional aggregates of cells derived from ES cells. When cells from embryoid bodies differentiated for 3–3.25 days were plated in methylcellulose medium supplemented with VEGF and SCF, blast cell colonies developed within 2–3 days. Upon transfer into liquid culture medium supplemented with growth factors, individual blast colonies could give rise to both haematopoietic and endothelial cells. Employing two distinct mouse ES cell lines, it has been shown that blast colonies are clonally derived but do not arise through cell migration and/or aggregation (Choi *et al.*, 1998).

The studies discussed in this section aimed to provide evidence for the existence of bipotent progenitors capable of haemato-endothelial differentiation. Unfortunately, the relationship of these cells to definitive HSCs has not been shown, and the emergence of definitive HSCs from haemangioblasts remains to be proven.

1.9.2. Haematogenic Endothelial Cells as HSC Lineage Precursors

The existence of haematogenic endothelium was suggested almost a century ago after endothelium-associated intra-aortic haematopoietic cell clusters had been described in different vertebrate embryos (Dantschakoff, 1909; Emmel, 1916; Jordan, 1917; Minot, 1912). The first experiments exploring the production of haematopoietic cells by the aortic endothelium were performed in the avian embryo. Labelling the vasculature of the chicken embryo with DiI-conjugated acetylated low-

density lipoproteins or nonreplicative *LacZ*-bearing retroviruses resulted in the formation of labelled intra-aortic haematopoietic cell clusters. It is not entirely clear whether only endothelial but not circulating haematopoietic cells were labelled at the beginning (Jaffredo et al., 2000; Jaffredo et al., 1998).

The existence of the haematogenic endothelium has been most convincingly shown in the zebrafish embryo. By employing in vivo time-lapse imaging of *Flk-1*, *c-Myb*, *CD41* and *Runx1* reporter transgenic zebrafish embryos, the production of haematopoietic cells by aortic endothelial cells has been visualised (Bertrand et al., 2010; Kissa and Herbomel, 2010). The haematogenic endothelium cells become evident around 30 hpf when they start to contract and bend towards the subaortic space. After 5–6 h, these cells loose contacts with adjacent endothelial cells, acquire haematopoietic progenitor morphology and enter first the subaortic space, then the axial vein and finally the circulation (Kissa and Herbomel, 2010). Since long-term tracing of these cells was not done, it needs to be established that they are indeed HSCs but not multipotent progenitors (Bertrand et al., 2010; Kissa and Herbomel, 2010).

To prove the existence of the haematogenic endothelium in the mouse embryo, *CD144* gene expression-specific Cre-mediated *in vivo* labelling was employed. In this experimental model, it has been found that the majority of haematopoietic cells in adult mice are labelled (Zovein *et al.*, 2008). Since mouse adult HSCs are $CD144^-$ (Taoudi *et al.*, 2005), the haematopoietic lineage labelling should occur during embryogenesis. Based on the assumption that CD144 is an exclusive endothelial

marker, which is the case in the adult organism (Lampugnani *et al.*, 1992), it was claimed that mouse definitive HSCs emerge from endothelial cells (Zovein *et al.*, 2010). However, in the mouse embryo, CD144 marks not only endothelial cells but also HSC lineage cells (North *et al.*, 2002; Rybtsov *et al.*, 2011; Taoudi *et al.*, 2008; Taoudi *et al.*, 2005). In fact, the labelling of haematopoietic lineage can be easily explained by CD144-specific Cre-mediated recombination in $CD144^{+}CD45^{-}CD41^{+}$ type I pre-HSCs or some earlier $CD144^{+}$ haematopoietic precursors (reviewed by Medvinsky *et al.*, 2011). However, Zovein *et al.* (2008) performed *in vitro* tamoxifen induction of Cre-ERT2 expressed under the control of the *CD144* gene promoter in embryonic liver and circulating cells, some of which are $CD144^{+}CD45^{+}$, and did not observe the labelling of haematopoietic cells due to the recombination of the *Rosa26R-LacZ* reporter gene. They concluded that the *CD144* gene is not transcribed in $CD144^{+}$ haematopoietic cells and that the CD144 protein is carried over from their endothelial ancestors.

However, studies on the specification of HSC lineage in the mouse embryo from our laboratory do not support the above conclusion. *Ex vivo* maturation of sorted $CD144^{+}CD45^{-}CD41^{+}$ type I pre-HSCs into $CD144^{+}CD45^{+}$ definitive HSCs through the intermediate $CD144^{+}CD45^{+}$ type II pre-HSC stage takes 4 days. By the end of this period, CD144 remains expressed on the surface of definitive HSCs at a high level, which is unlikely to be possible without *CD144* gene transcription. Furthermore, pre-HSC maturation into definitive HSCs involves several cell divisions, suggesting that the *CD144* gene should be transcribed in HSC lineage cells

in order to maintain the constant level of CD144 protein expression (Rybtsov *et al.*, 2011; Taoudi *et al.*, 2008).

The aim of another study employing *CD144* gene expression-specific Cre-mediated *in vivo* recombination was to establish the role of the *Runx1* gene in the development of mouse definitive HSCs and its link to the haematogenic endothelium. When *Runx1* was excised with *CD144* gene promoter-driven Cre, definitive HSCs in the mouse embryo did not develop. In contrast, *Runx1* deletion with Cre expressed in haematopoietic cells under the control of the *Vav1* gene promoter did not affect the development of mouse definitive HSCs (Chen *et al.*, 2009). Since *Runx1* ablation in haematopoietic cells does not affect the development of definitive HSCs, based on the assumption that CD144 is an exclusive endothelial marker it has been concluded that *Runx1* is required for the endothelio-haematopoietic transition during the production of definitive HSCs from the haematogenic endothelium (Chen *et al.*, 2009). However, *Runx1* excision affecting the development of definitive HSCs could also occur in CD144-expressing pre-HSCs and/or definitive HSCs (Rybtsov *et al.*, 2011; Taoudi *et al.*, 2008; Taoudi *et al.*, 2005). Although it is known that *Vav1* expression is restricted to haematopoietic cells (Ogilvy *et al.*, 1999), the evidence that the *Vav1* gene promoter is active in HSC lineage cells would support the idea that *CD144* gene expression-specific Cre-mediated *Runx1* deletion occurs prior $CD144^{+}$ cells acquire haematopoietic identity. In this case, the existence of the haematogenic endothelium would be very likely.

The existence of haematogenic endothelium has also been demonstrated *in vitro*. It has been shown using mouse ES cells. E-cadherin^{-}Flk-1^{+}CD144^{-} mesodermal cells, obtained during ES cell differentiation, gave rise to Flk-1^{+}CD144^{+}CD45^{-} endothelial cells capable of producing haematopoietic cells (Nishikawa *et al.*, 1998a). Importantly, cells with a similar phenotype and functional properties have been isolated from the day 9.5 mouse yolk sac and the embryo proper (Nishikawa *et al.*, 1998b). The above observations have been reinforced employing live cell imaging combined with single cell tracking (Eilken *et al.*, 2009).

Recently, a model combining both the haemangioblast and the haematogenic endothelium concepts has been proposed using the mouse ES cell differentiation model. Following the development of individual blast colonies using live cell imaging, it has been found that the blast colonies transform first into tight adherent Tie-2^{hi}c-Kit^{+}CD41^{-} endothelial cell colonies that then give rise to Tie-2^{-}CD41^{+} haematopoietic cells (Lancrin *et al.*, 2009). Thus, it is possible that haemangioblasts generate haematopoietic cells through the formation of haematogenic endothelial cells. Importantly, Tie-2^{hi}c-Kit^{+}CD41^{-} endothelial cells capable of producing haematopoietic cells *in vitro* can be found in the mouse embryo; however, it remains to be confirmed if these haematogenic endothelium cells originate from haemangioblasts.

As for the human embryo, the only attempt to demonstrate the haematogenic endothelium was made by employing *in vitro* haematopoietic differentiation of sorted CD34^{+}CD45^{-} cells considered to be endothelial (Oberlin *et al.*, 2002). As

discussed above, it has never been shown that $CD34^{+}CD45^{-}$ cells from the human dorsal aorta and the vitelline artery do not express such early haematopoietic markers as CD43 (Vodyanik *et al.*, 2006) or CD41 (Eilken *et al.*, 2009; Ferkowicz *et al.*, 2003; Lancrin *et al.*, 2009; Mikkola *et al.*, 2003); therefore, it is still not confirmed that in the human embryo endothelial cells give rise to haematopoietic cells.

Although some of the *in vitro* studies discussed above have convincingly shown that haematopoietic cells can be produced by endothelial cells (Eilken *et al.*, 2009; Lancrin *et al.*, 2009), the relationship of these endothelial and haematopoietic cells to definitive HSCs is not clear. The studies employing cell fate tracking upon *in vivo* labelling have not provided unambiguous evidence for the emergence of definitive HSCs from the haematogenic endothelium either. Thus, the haematogenic endothelium concept remains just one of the possible theories attempting to explain the embryonic development of definitive HSCs.

1.9.3. Mesodermal Cells as HSC Lineage Precursors

It is possible that the HSC lineage emerges not from haemangioblasts or haematogenic endothelium cells but directly from mesodermal precursors. It has been shown that in the mouse embryo between days 8.5 and 12 of development underneath the aortic floor persist the so-called subaortic patches formed by $Gata3^{+}$ mesodermal cells (Bertrand *et al.*, 2005; Manaia *et al.*, 2000). It was suggested that at day 10.5 of development the mesodermal cells give rise to c-$Kit^{+}AA4.1^{+}CD31^{+}CD41^{+}CD45^{-/lo}$ long-term repopulating haematopoietic cells detectable only by transplantation into immunodeficient adult recipient mice

(Bertrand *et al.*, 2005). These cells can be considered as pre-HSCs since they are not mature enough to repopulate wild-type hosts (reviewed by Medvinsky *et al.*, 2011). According to the model proposed by Bertrand *et al.* (2005), c-Kit^{+}AA4.1^{+}CD31^{+}CD41^{+}CD45$^{-/lo}$ pre-HSCs emerge in the subaortic patches and migrate towards the lumen of the dorsal aorta and form intra-aortic haematopoietic cell clusters where pre-HSCs mature into definitive HSCs, which are released into the circulation. The recent study on mouse embryonic pre-HSCs and definitive HSCs from our laboratory has suggested that the above scenario may be true. While definitive HSCs are localised to the endothelial lining of the dorsal aorta, pre-HSCs reside both in the luminal and subluminal layers of the dorsal aorta (Rybtsov *et al.*, 2011). The subaortic patches have also been described in the human embryo (Marshall *et al.*, 2000).

1.10. Dorso-Ventral Polarity in Haematopoietic Activity within the Embryonic Dorsal Aorta

In various vertebrate embryos, endothelium-associated haematopoietic cell clusters are found mainly on the ventral wall of the dorsal aorta (Emmel, 1916; Garcia-Porrero *et al.*, 1995; Jaffredo *et al.*, 1998; Jordan, 1917; Minot, 1912; Tavian *et al.*, 1996). Furthermore, a number of key genes responsible for definitive haematopoietic specification during embryonic development, such as *Runx1*, *Scl*, *c-Myb*, *Gata2*, *Gata3*, *Bmp4*, *Lmo2* and some others, are expressed in the ventral aortic endothelium and/or subaortic mesenchyme but not in the dorsal wall of the dorsal aorta (Bertrand *et al.*, 2005; Ciau-Uitz *et al.*, 2000; Durand *et al.*, 2007; Elefanty *et al.*, 1999; Manaia *et al.*, 2000; Marshall *et al.*, 2000; North *et al.*, 1999; Thompson *et al.*, 1998). These

observations led to functional testing of the dorso-ventral polarity in haematopoietic activity within the dorsal aorta and surrounding mesenchyme.

Subdissecting the day 11.5 mouse dorsal aorta and surrounding mesenchyme along the midline into the AoV and the AoD and assessing the number of committed haematopoietic progenitors in each of these two tissues, it was unexpectedly shown that CFU-Cs are uniformly distributed in the wall of the dorsal aorta. However, in the long-term repopulation assay, the vast majority of definitive HSCs were found in the AoV. Testing the mouse dorsal aorta at day 10.5 of development, before appearance of definitive HSCs, it has been shown that in the organ explant culture system the AoV but not the AoD is capable of HSC generation and expansion (Taoudi and Medvinsky, 2007).

At present, functional data on the existence of the dorso-ventral polarity in haematopoietic activity within the embryonic dorsal aorta are available only for the mouse embryo. Although *in vivo* imaging of the zebrafish embryo has provided rather convincing evidence that zebrafish definitive HSCs/multipotent progenitors emerge through the endothelio-haematopoietic transition of ventral aortic endothelial cells (Bertrand *et al.*, 2010; Kissa and Herbomel, 2010). Beside the observations of the distribution of intra-aortic haematopoietic cell clusters (Minot, 1912; Tavian *et al.*, 1996), no data to support the existence of the dorso-ventral polarity in haematopoietic activity within the human dorsal aorta has been presented so far.

1.11. Staging of Human Embryos

Performing research on the embryonic development of the human haematopoietic system, it is important to stage human embryos properly to divide this continuous process into consecutive phases. Different staging approaches are used by different researchers. Researchers having different backgrounds understand the term 'gestational age' differently. Embryologists by this term mean the postovulatory age of the human embryo (the age calculated based on the woman's last ovulation). Gynaecologists-obstetricians under this term understand the menstrual age of the human embryo (the age calculated from the beginning of the woman's last menstrual period). The difference between the two gestational ages is approximately two weeks; however, it cannot usually be determined precisely since the difference depends on the length of the woman's menstrual cycle, which is frequently variable. When research is performed on early human embryos, the discrepancy in age may be sufficient enough to corrupt the results severely. Therefore, an accurate staging system of human embryos should be used (O'Rahilly and Muller, 2000).

In present-day embryology, the Carnegie staging system, originally described by Streeter and refined by O'Rahilly and Muller (1987), is a gold standard to stage human embryos. Although this system has been accepted internationally and allows comparison between the findings of different researchers, the use of expressions as 'in a 22-day-old human embryo' or 'in a 6-week-old human embryo' are not uncommon (Huyhn *et al.*, 1995; Migliaccio *et al.*, 1986; Oberlin *et al.*, 2010; Oberlin *et al.*, 2002; Robin *et al.*, 2009; Tavian *et al.*, 1996). Often it is not even specified

whether this is the postovulatory gestational age or the postmenstrual gestational age (Robin *et al.*, 2009).

The Carnegie staging system covers the whole human embryonic period (the first 56 days of development), which starts with fertilisation and finishes with the onset of bone marrow formation in the humerus. The Carnegie staging system divides the human embryonic period into 23 Carnegie stages (CSs). Each CS is based on a complex of both external and internal morphological criteria and is not directly dependent on gestational age. Therefore, human embryos of the same gestational age can belong to different CSs, and vice versa, human embryos belonging to the same CS can be of different gestational ages. Embryos arranged only according to their gestational ages are not staged (O'Rahilly and Muller, 1987).

1.12. Immunophenotype of Human Adult HSCs

Haematopoiesis is usually considered as a hierarchy in which HSCs give rise to immature haematopoietic progenitors, which in turn give rise to committed haematopoietic progenitors producing mature haematopoietic cells. In this hierarchy, HSCs are the most challenging to study. The very low frequency of HSCs in haematopoietic tissue encourages the creation of methods of HSCs purification. Furthermore, some clinical applications require the isolation of HSCs. These questions can be addressed when the specific immunophenotype of human HSCs is defined. At present, no unique markers exist for human HSCs.

1.12.1. CD34

The most widely used positive marker for human HSCs is CD34. Civin *et al.* (Civin *et al.*, 1984) found this marker on approximately 1–2% of human bone marrow cells but not on peripheral mature haematopoietic cells. Berenson *et al.* (1988) showed that $CD34^+$ baboon bone marrow cells are able to reconstitute the haematopoietic system of lethally irradiated recipients. In contrast, animals transplanted with $CD34^+$ cell-depleted bone marrow were either nonengrafted or demonstrated bone marrow aplasia and pancytopenia. Later, the same research group showed that $CD34^+$ human bone marrow cells reconstitute myeloablated patients (Berenson *et al.*, 1991).

The $CD34^+$ cell population is very heterogeneous. There is one HSC within about 10000 $CD34^+$ cells sorted from human haematopoietic cell tissues. Only a small fraction of $CD34^+$ cells that are Lin^- (cells that do not express mature lineage markers such as CD2, CD3, CD4, CD8, CD11b, CD14, CD15, CD16, CD19, CD20, CD33, CD56, CD66b, CD123, CD235a) and $CD38^-$ possess HSC potential (reviewed by Bhatia *et al.*, 1997; Kondo *et al.*, 2003). In the umbilical cord blood, it has been shown that a population of approximately 600 human $Lin^-CD34^+CD38^-$ cells contains one HSC (Bhatia *et al.*, 1997).

For a long time, it was assumed that CD34 marks all human HSCs. However, it was shown that some human HSCs are $CD34^-$ (Bhatia *et al.*, 1998; Zanjani *et al.*, 1998). In umbilical cord blood, there is one $CD34^-$ HSC in 1×10^8 mononuclear cells compared with one $CD34^+$ HSC in 1×10^6 mononuclear cells (Bhatia *et al.*, 1998). It was described that umbilical cord blood Lin^-CD34^- cells, which do not initially have

or have extremely low HSC potential, do acquire it after four days of coculture with bone marrow stromal cells, which coincides with their conversion to a $CD34^+$ phenotype (Nakamura *et al.*, 1999). Therefore, it was suggested that $CD34^-$ HSCs are upstream of $CD34^+$ HSCs and that patients transplanted with $CD34^+$ cells do not receive essential $CD34^-$ HSCs and may experience graft failure. Kato *et al.* (Kato *et al.*, 2001) demonstrated that $CD34^-$ bone marrow cells from patients transplanted with sorted $CD34^+$ HSCs do not have potential to mature into $CD34^+$ cells. In contrast, $CD34^-$ bone marrow cells from healthy volunteers or from patients transplanted with unmanipulated bone marrow cells or T cell-depleted bone marrow possess this potential. The clinical significance of this finding remains to be clarified.

1.12.2. CD133

It was proposed that CD133 can provide an alternative to CD34 for the selection of human HSCs. CD133 is expressed on 20–60% of $CD34^+$ cells isolated from adult and foetal bone marrow, foetal liver, umbilical cord blood and mobilised peripheral blood. On $CD34^+$ cells obtained from adult haematopoietic tissues, the expression of CD133 is higher compared to foetal ones (Yin *et al.*, 1997). The depletion of Lin^+ and $CD38^+$ cells from the umbilical cord blood $CD34^+$ cell population increases the proportion of $CD133^+$ up to almost 90%. It is important that on $CD34^-$ cells CD133 is expressed at a very low level ($CD133^+$ cells are not detectable within the $CD34^-$ cell population and only 0.2% of cells within the $Lin^-CD34^-CD38^-$ cell population express CD133) (Gallacher *et al.*, 2000).

It has been shown that $CD34^+CD133^+$ cells can provide long-term multilineage human haematopoietic cell engraftment in foetal sheep and irradiated NOD/SCID mice. It seems that CD133 marks all HSCs within the $Lin^-CD34^-CD38^-$ cell population. It was proposed that CD133 can be used instead of CD34 as a 'unifying' marker of $CD34^+$ and $CD34^-$ HSCs. However, it has been shown that some cells within the $CD34^+CD133^-$ cell population have HSC potential. Thus, CD133 does not mark all human HSCs (Gallacher *et al.*, 2000; Yin *et al.*, 1997).

1.12.3. Thy-1

Human $Lin^-CD34^+Thy\text{-}1^+$ but not $Lin^-CD34^+Thy\text{-}1^-$ cells are the only cells in adult and foetal bone marrow, foetal liver, umbilical cord blood and mobilised peripheral blood that are able to sustainably engraft irradiated SCID-hu mice (SCID mice transplanted with human foetal organs, including thymus, liver, bone with cavity, spleen and/or lymph nodes) (Baum *et al.*, 1992). In clinical settings, it has been shown that mobilised peripheral blood $CD34^+Thy\text{-}1^+$ cells with or without lineage depletion can reconstitute the haematopoietic system of preconditioned patients (Negrin *et al.*, 2000).

1.12.4. c-Kit

The distinction between $c\text{-}Kit^+$ and $c\text{-}Kit^-$ cells within human haematopoietic tissue $CD34^+$ cell population is arbitrary since c-Kit is expressed on these cells in a continuum. Often, the $c\text{-}Kit^-$, the $c\text{-}Kit^{low}$ and the $c\text{-}Kit^{high}$ cell populations are gated. It was demonstrated that human HSCs are predominantly $CD34^+c\text{-}Kit^{low}$. Using the foetal sheep xenotransplantation model, 3000 to 5000 $CD34^+c\text{-}Kit^{low}$ cells were

sufficient to show long-term multilineage engraftment of human haematopoietic cells in recipients. Due to a relatively small number of recipients used in the study, limiting dilution analysis was not possible (Kawashima *et al.*, 1996).

1.12.5. KDR (Flk-1)

It has been reported that approximately 100 times higher enrichment for human HSCs compared with the $Lin^-CD34^+CD38^-$ cell population can be achieved by isolating KDR^+ cells (Ziegler *et al.*, 1999). It has been shown that $CD34^+KDR^+$ but not $CD34^+KDR^-$ cells from human bone marrow, peripheral blood, mobilised peripheral blood and umbilical cord blood can generate long-term multilineage haematopoietic cell engraftment in irradiated NOD/SCID mice. It was estimated that there is one HSC in five $CD34^+KDR^+$ cells. This cell population is Lin^- and represents about 0.1 to 0.5% of $CD34^+$ cells and about 1 to 2% of $Lin^-CD34^+CD38^-$. Only around 30% of $CD34^+KDR^+$ cells are $CD38^-$. This suggests that depletion of $CD38^+$ cells would further enrich this cell population for human HSCs. KDR is also expressed on human HSCs within Lin^-CD34^- cell population (Ziegler *et al.*, 1999). Surprisingly, following the report that KDR allows human HSC enrichment to a very high level, the marker has not been widely, if at all, employed.

1.12.6. CD143 (Angiotensin-Converting Enzyme)

Recently, it has been shown that CD143 marks human HSCs within the $CD34^+$ cell population (Jokubaitis *et al.*, 2008). $CD34^+CD143^+$ but not $CD34^+CD143^-$ cells from umbilical cord blood generate long-term multilineage haematopoietic cell engraftment in irradiated NOD/SCID mice. Limiting dilution analysis of long-term

xenotransplantation data demonstrates that about 3000 $CD34^+CD143^+$ cells sorted from human umbilical cord blood contain one HSC (Jokubaitis *et al.*, 2008). Interestingly, the previously described $CD34^+CD45^+$ cell clusters on the ventral wall of the human embryonic dorsal aorta express CD143 (Sinka *et al.*, 2012; Tavian *et al.*, 1996).

1.12.7. Hoechst 33342 Efflux

Goodell *et al.* (1996) reported that staining mouse bone marrow cells with the fluorescent dye Hoechst 33342 allows isolating in a single step a cell population that is highly enriched for HSCs. Hoechst 33342^- cell population contains HSCs and is referred to as a 'side population'. For mouse haematopoietic tissues, the side population assay allows over 1000-fold enrichment for HSC activity when compared to unmanipulated bone marrow. Although not all of the mouse HSCs belong to the side population, Hoechst 33342^+ cells have approximately 500 times lower HSC activity than side population cells.

The side population assay has also been applied to human haematopoietic tissues. Unlike mouse haematopoietic side population cells, human Hoechst 33342^- haematopoietic cells constitute a much more phenotypically and functionally heterogeneous population. For example, low-density human foetal liver cells contain around 0.15% of side population cells. Only about 20% of these cells are $CD34^+$. Most of the side population cells, both $CD34^+$ and $CD34^-$ cells, express at least one of the lineage markers. Although all of the human HSCs present in foetal liver were shown to be Hoechst 33342^-, due to significant heterogeneity it is not possible to

enrich the side population of human haematopoietic cells for HSC activity without prior depletion of Lin^+ and $CD38^+$ cells and enrichment for $CD34^+$ cells. When human $Lin^-CD34^+CD38^-$ foetal liver cells are depleted from Hoechst 33342^+ cells, the frequency of HSCs increases only tenfold, but due to the toxic effect of exposing the cells to Hoechst 33342 there is observed an approximately threefold loss of HSCs (Uchida *et al.*, 2001).

1.12.8. High Cytosolic Aldehyde Dehydrogenase Activity

Cytosolic aldehyde dehydrogenase (ALDH) is expressed at high levels in HSCs, as has been demonstrated first in the mouse. After counterflow centrifugal elutriation and Lin^+ cell depletion, mouse bone marrow cells were incubated with a fluorescent ALDH substrate dansyl aminoacetaldehyde, which diffuses across the cell membrane and after oxidation by ALDH forms dansyl glycine is retained intracellularly. $ALDH^+$ cells were identified by dansyl fluorescence. Every tenth cell in this population was shown to be an HSC (Jones *et al.*, 1996).

More recently, a newer fluorescent ALDH substrate BODIPY aminoacetaldehyde (Aldefluor) was used to select human HSCs. Both HSCs and haematopoietic progenitors reside within a fraction of human umbilical cord blood cells that exhibits low light scatter (SSC) properties, absence of the lineage markers and high expression of ALDH. HSCs were found predominantly in $Lin^-SSC^{lo}CD34^+ALDH^+$ cell population. Limiting dilution analysis estimated the frequency of HSCs to be 1 in 4687 cells of this phenotype, which was approximately sevenfold higher than within Lin^-CD34^+ cell population. Within $Lin^-SSC^{lo}CD34^+ALDH^-$ cell population,

less than one HSC per 45000 cells was found. $Lin^{-}SSC^{lo}CD34^{-}ALDH^{+}$ cells failed to generate long-term multilineage haematopoietic cell engraftment in irradiated NOD/SCID mice. This suggests that the majority of human HSCs are enriched within the cell population that expresses both CD34 and ALDH (Storms *et al.*, 2005).

1.12.9. Most Precise Immunophenotype of Human Adult HSCs to Date

Studying HSCs derived from umbilical cord blood, Majeti et al. (2007) have isolated HSC activity within the $Lin^{-}CD34^{+}CD38^{-}Thy\text{-}1^{+}CD45RA^{-}$ cell population to as few as 10 cells. Later, this immunophenotype was confirmed (Doulatov et al., 2010) and an additional HSC marker, CD49f, was proposed (Notta et al., 2011). This allowed separating HSCs and transiently engrafting multipotent haematopoietic progenitors. Although CD49f is expressed by 30–35% of $Lin^{-}CD34^{+}CD38^{-}Thy\text{-}1^{+}CD45RA^{-}$ cells, limiting dilution analysis has shown that the frequency of HSCs within the $Lin^{-}CD34^{+}CD38^{-}Thy\text{-}1^{+}CD45RA^{-}CD49f^{+}$ cell population is still one in 10 cells (Notta et al., 2011). The use of more receptive for human HSCs neonatal but not adult NSG recipient mice by Majeti et al. (2007) may explain the discrepancy. Importantly, considerable HSC activity was also detected within Thy-1- cell population, which previously was considered as harbouring exclusively multipotent progenitors (Majeti et al., 2007). Approximately 4.5% of cells within the $Lin^{-}CD34^{+}CD38^{-}Thy\text{-}1^{-}CD45RA^{-}CD49f^{+}$ cell population are HSCs (Notta et al., 2011). This highlights that human HSCs are heterogeneous and no unique marker or marker combination have been found for these cells so far.

1.13. Summary and the Goal of the Present Study

HSCs can only be truly assessed by using the long-term repopulation assay. Until now, the development of the haematopoietic system in the early human embryo was studied exclusively *in vitro*; therefore, only the emergence of cells with HSC immunophenotype, which are not necessarily HSCs, has been assessed. Considering this and the potential impact that a human subject study may have on the development of novel therapeutic strategies, the main goal of the present study was to elaborately describe the development of the earliest HSCs in the human embryo. The results of this study are organised in three subchapters:

1. HSCs in the Early Human Embryo. Here, I describe the spatio-temporal distribution of HSC in the early human embryo and provide evidence for their stemness.
2. Regenerative Potential of the Early Human Embryonic HSCs. In this result subchapter, I compare the regenerative potential of early human embryonic HSCs to that of HSC from other sources.
3. Localisation and Immunophenotypic Characterisation of Human AGM Region HSCs. Here, I describe the dorso-ventral polarity in HSC activity within the human AGM region and provide the first data on the immunophenotype of the earliest human HSCs.

2. MATERIALS AND METHODS

2.1. General Buffers

Buffer 1 (dissection buffer) is a room temperature Dulbecco's phosphate buffered saline (DPBS) with Ca^{2+} and Mg^{2+} (Sigma-Aldrich) containing 7% of heat-inactivated foetal bovine serum (FBS) (PAA Laboratories) and 100 IU/ml of penicillin and 100 μg/ml of streptomycin (Pen Strep solution from Invitrogen).

Buffer 2 (dissociation buffer) is an ice-cold DPBS without Ca^{2+} and Mg^{2+} containing 7% of heat-inactivated FBS, 100 IU/ml of penicillin and 100 μg/ml of streptomycin.

Buffer 3 (flow cytometry buffer) is an ice-cold DPBS without Ca^{2+} and Mg^{2+} containing 2% of heat-inactivated FBS with or without 100 IU/ml of penicillin and 100 μg/ml of streptomycin (the antibiotics were added in the case of a cell sorting).

2.2. Source and Processing of Human Embryonic, Foetal and Neonatal Tissues

Human embryonic and foetal tissues were obtained immediately after elective termination of pregnancy. The procedure was carried out by medical professionals at the Royal Infirmary of Edinburgh, Edinburgh, Scotland, UK using mifepristone and misoprostol. Human umbilical cord blood samples were provided by the Scottish National Blood Transfusion Service (frozen mononuclear cells) and the Edinburgh Reproductive Tissue Biobank (full blood). In both cases, the samples were collected from full-term umbilical cords and placentas. The study was approved by the Lothian Research Ethics Committee (reference nos. 08/S1101/1 and 09/S1102/35). Before

tissue specimens were obtained and anonymised, each patient gave informed consent in writing for the use of human embryonic and foetal tissues in research.

The developmental stage of human embryos was determined according to the Carnegie staging system (O'Rahilly and Muller, 1987). The postmenstrual gestational age of human foetuses was determined by obstetrician-gynaecologists using medical ultrasonography.

Embryonic and foetal tissue dissection was performed in buffer 1. Whenever possible, my goal was to obtain from the same human embryo the AGM region, the yolk sac, the liver, the umbilical cord and the placenta, which were dissected under a Leica MZ8 stereomicroscope (Leica) using electrolysis-sharpened tungsten needles. In some cases, it was not possible to obtain all the above mentioned tissues as specimens were damaged. The dissection procedure was similar to that previously described for the mouse embryo and is described below (Medvinsky *et al.*, 2008).

For the enzymatic treatment of the human AGM region, yolk sac, liver and umbilical cord, 1 mg/ml of collagenase/dispase (Roche) and 0.12 mg/ml of DNase I (Roche) were added to tissues suspended in buffer 1 and incubated at 37° C for 40 min with gentle shaking. For the enzymatic treatment of placental tissue, two previously published protocols were employed with slight modifications (Barcena *et al.*, 2009; Robin *et al.*, 2009). After placentas were dissected free from maternal tissues and blood clots, they were minced with a surgical scissors. Then, either 181 U/ml of type I-A collagenase (Sigma-Aldrich), 0.7 mg/ml of type I-S hyaluronidase (Sigma-

Aldrich) and 0.12 mg/ml of DNase I (Barcena *et al.*, 2009) or 1 mg/ml of collagenase/dispase, 3 mg/ml of pancreatin (Sigma-Aldrich) and 0.12 mg/ml of DNase I (Robin *et al.*, 2009) were added to placental tissues suspended in buffer 1 and incubated at 37° C for 60 min with gentle shaking. At the end of enzymatic treatment, tissue digests were washed with buffer 2. In all instances, cells were centrifuged at 330×g for 5 min. For preparation of placental cells, in contrast to other tissues, 2 mM of EDTA (Sigma-Aldrich) were added to buffer 2. After the first wash with buffer 2, tissue digests were pipetted gently to prepare a single cell suspension. Human AGM region, yolk sac, liver and umbilical cord cells were washed an additional time with buffer 2. To remove cell clumps, placental cells were passed through a 40 μm nylon cell strainer (BD) and washed four more times. Finally, cells were resuspended in buffer 2 and kept on ice until transplantation, which was performed within 3–4 h of termination of pregnancy. In the majority of experiments with the human placenta, I omitted Ficoll-Paque density gradient separation step following the obtaining of a single cell suspension to minimise possible cell losses.

Full umbilical cord blood obtained from the Edinburgh Reproductive Tissue Biobank was diluted 1:4 with buffer 2 supplemented with 2 mM of EDTA and layered over Ficoll-Paque (StemCell Technologies) and processed according to manufacturer's instructions to separate mononuclear cells. Frozen mononuclear cells obtained from the Scottish National Blood Transfusion Service were removed from the liquid nitrogen, immediately thawed in a water bath at 37° C and washed twice in buffer 2 supplemented with 2 mM of EDTA. Then, $CD34^+$ cells were isolated employing the

Miltenyi Biotec magnetic cell separation technique. Finally, cells were resuspended in buffer 2 and kept on ice until transplantation.

2.3. Xenogeneic Long-Term Repopulation Assay

NSG mice (Shultz *et al.*, 2005) were used as recipients for human HSCs. Animals were bred within the University of Edinburgh, Edinburgh, Scotland, UK according to the provisions of the Animal (Scientific Procedures) Act 1986 under the project license granted by the Home Office. All researchers who performed experiments with the mice held personal licenses granted by the Home Office.

Mice were kept under specific pathogen-free conditions in isolators or individually-ventilated cages. Autoclaved drinking water and γ-irradiated chow diet was provided *ad libitum*. During the first 4 weeks after irradiation, autoclaved drinking water was supplemented with 0.1 mg/ml of enrofloxacin (10% Baytril solution from Bayer) or 1.67 mg/ml of neomycin (Invitrogen). Animals were exposed to 14-h light and 10-h dark cycle.

Up to 6 h before transplantation with human cells, 6–8-week-old female NSG mice received a sublethal total body irradiation dose of 3.5 Gy at a rate of 0.75 Gy/min from a ^{137}Cs source (GSR D1 γ-irradiator, Gamma-Service Medical). Animals were transplanted with cells intravenously via the tail vein. Some xenotransplantation experiments were performed by Dr Stanislav Rybtsov. The number of transplanted human embryonic cells were expressed in embryo equivalents (e.e.), defined as a unit of cells equivalent to the number of cells present in one tissue. Starting from week 6–

8 after transplantation, NSG recipient mice were bled by tail vein nicking every 1–2 months. Human HSC contribution was assessed by flow cytometry analysis cell immunolabelling with anti-human CD45 antibody. At the end of each experiment (usually 4–8 months after transplantation), all recipients (including those found nonengrafted after peripheral blood analysis) were killed by dislocation of the neck according to the Schedule 1 of the Animal (Scientific Procedures) Act 1986, and their bone marrow was collected. Bone marrow cells were labelled with anti-human CD45 antibody, and flow cytometry analysis was performed to detect the presence of human haematopoietic cells. The peripheral blood, bone marrow, spleen and thymus of engrafted NSG recipient mice were further analysed by flow cytometry analysis. The bone marrow and spleen from primary recipients were used in secondary transplantation experiments. On many occasions, xenotransplantation experiments in conjunction with the limiting dilution assay were also performed.

2.4. Syngeneic Long-Term Repopulation Assay

B6.SJL-*Ptprc*^{a} *Pepc*^{b}/Boy mice (Ly5.1/Ly5.1) were used as recipients for HSCs from C57BL/6 mouse embryos (Ly5.2/Ly5.2) and bone marrow 'carrier' cells from F1 hybrid mice (Ly5.1/Ly5.2). Ly5.1/Ly5.1, Ly5.2/Ly5.2 and Ly5.1/Ly5.2 mice were bred and kept under conditions similar to those for NSG mice, with the only difference being that the immunocompetent mice were housed in open-top cages.

Up to 6 h before transplantation with mouse cells, 6–8-week-old female Ly5.1/Ly5.1 mice received a split sublethal total body irradiation dose of 9.5 Gy (5 Gy and 4.5 Gy 3 h apart) at a rate of 0.59 Gy/min from a ^{137}Cs source. Animals were transplanted

with cells intravenously via the tail vein. The dose of transplanted mouse embryonic cells was expressed in e.e. At week 8 after transplantation, Ly5.1/Ly5.1 recipient mice were bled by tail vein nicking. Donor HSC contribution was assessed by flow cytometry analysis following cell immunolabelling with anti-mouse CD45.1 and CD45.2 monoclonal antibodies.

2.5. Analysis of NSG Recipient Mouse Tissues

To collect recipient mouse tissues, buffer 2 supplemented with 2 mM of EDTA was used. NSG mice transplanted with human AGM region, yolk sac, liver, umbilical cord, placental or bone marrow cells from primary recipients were bled by tail vein nicking, and erythrocytes were lysed using BD Pharm Lyse Buffer (BD) according to manufacturer's instructions. To analyse haematopoietic tissues, the spleen, thymus, long bones, coxal bones and sternum were obtained. Spleen and thymus were gently mashed in a small volume of the buffer through a 40 μm nylon cell strainer. The bone marrow was flushed out from the bones, and a single cell suspension was prepared by gentle pipetting. A cell fraction associated with endosteum was obtained from bones after treatment with 1 mg/ml of collagenase/dispase and 0.12 mg/ml of DNase I at 37° C for 40 min with gentle shaking and added to the flushed-out bone marrow fraction. The cells were spun down, resuspended in buffer 2 and kept on ice until transplantation. To calculate the proportion of total bone marrow transplanted into secondary recipients, we used previously reported data on the distribution of bone marrow cells in different mouse bones (Boggs, 1984).

2.6. Human CFU-C Assay

Human CFU-C number in dissociated human embryonic tissues was detected by plating 0.1–0.05 e.e. of cells into the MethoCult H4034 Optimum Medium (StemCell Technologies) following manufacturer's recommendations. Human CFU-Cs in the bone marrow of NSG recipient mice engrafted with human HSCs were detected by plating 10000–25000 bone marrow nucleated cells. The bone marrow from a nontransplanted NSG mouse was used as a negative control. Cells were incubated at 37° C, in 5% CO_2 and ≥95% humidity for 14 days. Haematopoietic colonies were enumerated microscopically. Some individual colonies were subject for cytospin analysis (May-Grunwald-Giemsa staining) and flow cytometry analysis (immunolabelling with anti-human CD45 and CD235a and anti-mouse CD45 and Ter119 antibodies).

2.7. Cytospin Preparation and May-Grunwald-Giemsa Staining

Fourteen days after the human CFU-C assay had been set up, individual haematopoietic colonies were harvested with a pipette under direct microscopic visualisation. Cells were washed from methylcellulose medium three times with buffer 3 and resuspended in 100 µl of the same buffer. Then, cells were loaded into individual cytospin chambers (Shandon) placed on top of a filter card (Thermo) and a poly-L-lysine-coated microscope slide (VWR International) and spun in a Cytospin 3 centrifuge (Shandon) at 1000 rpm for 5 min. Microscope slides were removed from the cytospin chambers, air-dried, fixed with 100% methanol at room temperature for 1 min and air-dried again.

To stain cytospins, May-Grunwald-Giemsa staining was employed. May-Grunwald stain (BDH Gurr) and Giemsa stain (BDH Gurr) stock solutions were diluted with DPBS without Ca^{2+} and Mg^{2+} 1:1 and 1:9, respectively. Both working solutions were prepared immediately before cell staining and were filtered through grade 1 filter paper (Whitman). Methanol-fixed cells were first stained with May-Grunwald stain for 14 min and then with Giemsa stain for 12 min. After this, microscope slides were washed with running tap water, immersed in distilled water and air-dried. Finally, cells were mounted in DPX mounting medium (Agar Scientific) and covered with a cover slip (VWR International). Cytospins were analysed next day using an Olympus BX61 compound microscope (Olympus).

2.8. Flow Cytometry Analysis and FACS

The following mouse anti-human monoclonal antibodies (all from BD) were used for flow cytometry analysis and/or FACS: CD3-APC, PE and PerCP (clones SK7 and SP34-2), CD4-APC and APC-Cy7 (clone RPA-T4), CD8-PE and PE-Cy7 (clone RPA-T8), CD11b-PE-Cy7 (clone ICRF44), CD13-APC (clone WM15), CD14-APC and APC-Cy7 (clones M5E2 and MφP9), CD19-PE (clone HIB19), CD33-PE (clone WM53), CD34-APC (clone 8G12), CD38-FITC and PE (clone HIT2), CD41a-FITC (clone HIP8), CD45-Biotin, FITC and V450 (clone HI30), CD66b-FITC (clone G10F5), CD94-APC (clone HP-3D9), CD235a-APC (clone GA-R2), IgM-Biotin (clone G20-127), αβ TCR-FITC (clone WT31) and γδ TCR-PE (clone 11F2). Mouse anti-human CD144-PE monoclonal antibody (clone TEA 1/31) was purchased from Beckman Coulter. Mouse anti-human c-Kit-APC (clone A3C6E2), Thy-1-APC (clone DG3) and CD105-APC (clone 43A4E1) monoclonal antibodies were a kind

gift from Miltenyi Biotec. Mouse anti-human CD45RA-eFluor 450 (clone HI100) and mouse anti-mouse CD45.1-APC (clone A20) and CD45.2-PE (clone 104) monoclonal antibodies were bought from eBioscience. Appropriate isotype controls, fluorochrome-conjugated streptavidin reagents and rat anti-mouse CD45-APC and FITC (clone 30-F11), Ter119-FITC and PE and in-house biotin-conjugated CD144 (clone 11D4.1) monoclonal antibodies were purchased from BD. The Human FcR Blocking Reagent (Myltenyi Biotec) and anti-mouse CD16/32 purified monoclonal antibody (clone 93) (eBioscience) were used to prevent unwanted binding of antibodies to Fc receptors. All the antibodies and reagents listed above were used at final concentrations either recommended by manufacturers or determined by titration in-house.

Cell immunolabelling and washes were performed in buffer 3. Up to 1×10^6 cells were resuspended in 50 µl of the Human FcR Blocking Reagent and anti-mouse CD16/32 purified monoclonal antibody diluted 1:10 and 1:200, respectively, in buffer 3 and incubated on ice for 10 min. Then, 50 µl of antibody solution at the double concentration were added to the cells, making the final cell suspension volume to 100 µl. Cells were stained on ice and in the dark for 30 min. After this, cells were washed twice. If required, secondary staining with fluorochrome-conjugated streptavidin was performed in a similar way. Otherwise, cells were resuspended in buffer 3 supplemented with 1.5 µg/ml of 7-amino-actinomycin (7-AAD) (eBioscience) and proceeded for flow cytometry analysis or FACS.

A FACSCalibur, LSRFortessa (both from BD) or CyAn ADP (Dako) instruments were used for flow cytometry analysis. FACS and postsort purity checks were performed on a FACSAria II (BD) or Celula mvs360b (Celula) instruments. Sorted cells were spun down, resuspended in buffer 2 and kept on ice until transplantation. Flow cytometry data were analysed with FlowJo v7.6.1 software (Tree Star).

2.9. Magnetic Cell Separation

The magnetic cell separation technology developed by Miltenyi Biotec was employed to isolate $CD34^+$ cells from human umbilical cord blood samples and $CD45^+$ and $CD144^+$ cells from human AGM region cell samples. All reagents and materials listed below were purchased from Miltenyi Biotec. Human CD34 and CD45 MicroBeads were used to enrich for $CD34^+$ and $CD45^+$ cells. $CD144^+$ cells were isolated using anti-PE MicroBeads following cell immunolabelling with mouse anti-human CD144-PE monoclonal antibody (clone TEA 1/31) (Beckman Coulter). In all cases, magnetic cell separation was performed manually using a QuadroMACS separator and LS columns. Cell immunolabelling, washes and isolation were performed in buffer 3 according to manufacturer's recommendations.

2.10. Confocal Microscopy Analysis of the Mouse Bone Marrow

Femurs were dissected from NSG recipient mice engrafted with human HSCs and immediately fixed in 10% neutral buffered formalin (Sigma-Aldrich). After 2 h, bones were decalcified in 0.4 M EDTA solution (pH=7.2) for 4 days at 4° C. Then, femurs were transferred into 30% sucrose (Sigma-Aldrich) solution in DPBS without Ca^{2+} and Mg^{2+} and, after bones had sunk, into a 1:1 mixture of 30% sucrose solution

and OCT embedding medium (CellPath) and kept at 4° C for 1 day. For cryosectioning, bones were snap-frozen in OCT embedding medium on dry ice. Tissue sections were cut using a Leica CM1900 cryostat (Leica), transferred onto poly-L-lysine-coated microscope slides and kept at -20° C until immunolabelling. Individual tissue sections were encircled using a hydrophobic barrier pen (Vector Labs) and rehydratated in buffer 3 for 1 min. For antigen retrieval, tissue sections were incubated in 10 mM sodium citrate (Sigma-Aldrich) and 0.05% Tween 20 (Sigma-Aldrich) buffer (pH=6) at 80° C for 20 min followed by a wash in buffer 3 for 5 min. Then, the Human FcR Blocking Reagent and anti-mouse CD16/32 purified monoclonal antibody diluted 1:10 and 1:200, respectively, in buffer 3 were added to tissue sections. After 10 min, this solution was discarded and mouse anti-human CD45-FITC (clone HI30, BD) and unlabelled rat anti-mouse CD45 (clone 30-F11, BD) antibodies diluted 1:10 and 1:100, respectively, in buffer 3 were added to tissues sections and incubated for 1 h in the dark followed by three washes, 5 min each, in buffer 3. After this, goat anti-rat IgG-Alexa Fluor 546 antibody (Invitrogen) diluted 1:100 in buffer 3 was added to tissue sections and incubated for 30 min in the dark followed by three washes, 5 min each, in buffer 3. Then, nuclei were stained by incubating tissue sections in buffer 3 containing 0.2% of DAPI (Invitrogen) for 10 min in the dark followed by three washes, 5 min each, in buffer 3. Finally, tissue sections were mounted in VECTASHIELD mounting medium (Vector Labs) and covered with a cover slip. Microscope slides were analysed next day using Leica DM IRE2 confocal microscope (Leica).

2.11. DNA Fingerprinting

Short tandem repeat (STR) analysis to discriminate between embryonic versus maternal origin of haematopoietic repopulation was performed by Dr Lindsey Welch at the University of Strathclyde, Glasgow, Scotland, UK. Genomic DNA was extracted from human embryonic or foetal donor tissues and from peripheral blood, bone marrow and/or spleen cells obtained from corresponding NSG recipient mice repopulated with human haematopoietic cells. For this, the QIAamp DNA Investigator Kit (Qiagen) and a QIAcube robotic instrument (Qiagen) were used. DNA quantification was performed with the Plexor HY System (Promega) and an Mx3500P real-time PCR machine (Stratagene). DNA quantification values were determined using the Plexor Data Analysis Software (Promega). DNA samples and PCR positive and negative controls were amplified with the AmpFlSTR SGM Plus PCR Amplification Kit (Applied Biosystems). PCR products were detected on a 3130 Genetic Analyzer (Applied Biosystems). Handling of raw data and genotyping were carried out with GeneMapper ID v3.2.1 software (Applied Biosystems). All the reagents and instruments listed above were used according to manufacturers' recommendations. DNA match probabilities were calculated based on allele frequencies in the Scottish Caucasian population.

2.12. Statistical Analyses

Data are presented as a mean±standard deviation (SD) or a 95% confidence interval (CI). Correlation between variables was assessed based on the Pearson product-moment correlation coefficient or the Spearman rank correlation coefficient and two-tailed test of significance. Selection of parametric versus nonparametric tests to

perform comparison between groups was based on the evaluation of normality using the Kolmogorov-Smirnov test. If data sets were normally distributed, comparison between groups was performed using the Independent-Samples T Test or the Paired-Samples T Test. In the opposite case, the Mann-Whitney U Test or the Wilcoxon Signed Ranks Test were used. Statistical significance was attributed to probability value $P<0.05$. For the above statistical procedures, SPSS 15.0 for Windows software (SPSS) was used. Validity tests for the single-hit Poisson model and the limiting dilution analysis were performed using ELDA software (Hu and Smyth, 2009) available at http://bioinf.wehi.edu.au/software/elda/.

3. RESULTS

3.1. HSCs in the Early Human Embryo

3.1.1. Introduction

It was long assumed that in vertebrate embryos the yolk sac is the unique provider of definitive HSCs (Moore and Metcalf, 1970; Moore and Owen, 1965; Moore and Owen, 1967). A paradigm shift occurred after the experiments with chimeric quail and chicken embryos had suggested the intra-embryonic origin of HSCs (Dieterlen-Lievre, 1975). Shortly after, the same was confirmed for the amphibian embryo (Turpen *et al.*, 1981). In the mouse embryo, the first HSCs were also detected in the intra-embryonic AGM region (Muller *et al.*, 1994). Moreover, it has been shown that the mouse AGM region initiates and sustains the generation of definitive HSCs independently of the yolk sac (Medvinsky and Dzierzak, 1996). Considering that the qualitative and the quantitative assessment of HSCs can usually be performed employing the *in vivo* long-term repopulation assay (Jones *et al.*, 1990; Ploemacher and Brons, 1989; Spangrude *et al.*, 1988; Szilvassy *et al.*, 1990), nothing has been reported regarding the development of HSCs in the human embryo. The fundamental understanding of this process is a prerequisite for the development of novel therapeutic strategies such as a guided *in vitro* differentiation of human pluripotent stem cells into transplantable HSCs and a controlled *ex vivo* expansion of human umbilical cord blood and adult bone marrow HSCs.

Until now, the early embryonic development of the human haematopoietic system has been studied mainly by immunohistochemical methods and *in vitro* assays. Similar to other vertebrate embryos, in the human embryo, haematopoietic cells are

generated both in the yolk sac and in the embryo proper (Huyhn *et al.*, 1995). In the yolk sac, the first haematopoietic cells, primitive erythroid cells, are produced in blood islands between days 16 and 18.5 of development (Bloom and Bartelmez, 1940; Luckett, 1978). As for intra-embryonic haematopoiesis, some endothelium-associated $CD34^{+}CD45^{+}$ haematopoietic cell clusters appear on the ventral wall of the human dorsal aorta between days 26 and 37 of development (Minot, 1912; Tavian *et al.*, 1996; Tavian *et al.*, 1999). This territory has never been tested for HSC activity, but it has been claimed that the first human HSCs emerge within these cell clusters. Later, it has been demonstrated that the AGM region contains haematopoietic progenitors able to give rise to myeloid, B, T and NK cells, whereas the yolk sac haematopoietic progenitors were able to produce myeloid and NK cells but not B and T cells (Tavian *et al.*, 2001). Despite the fact that these data were obtained *in vitro*, it is speculated that the earliest human HSCs emerge in the AGM region as only this tissue showed unrestricted haematopoietic differentiation potential. Due to the lack of *in vivo* data, it is still not clear where and when the first HSCs arise in the human embryo.

3.1.2. Experimental Approach

Early human embryos were obtained immediately after elective termination of pregnancy. In the majority of cases, it was possible to dissect from the same embryo all tissues that, based on studies in the mouse, are considered to be involved in the embryonic development of the mammalian haematopoietic system (reviewed by Medvinsky *et al.*, 2011). This approach in a combination with access to human embryos at different developmental stages allowed establishing the spatio-temporal

distribution of HSCs in the early human embryo. To assess HSC activity, single cell suspensions prepared from different dissected embryonic tissues were individually transplanted into sublethally irradiated NSG mice. In all experiments, female recipient mice were used since they are more receptive for human HSCs than male recipient mice (Notta *et al.*, 2010). Human HSCs were detected by their capacity to provide long-term (>4 months) multilineage haematopoietic engraftment of human origin and to self-renew upon intravenous transplantation into sublethally irradiated NSG mice.

3.1.3. Spatio-Temporal Distribution of HSCs in the Early Human Embryo

To establish the spatio-temporal distribution of HSCs in the early human embryo, transplantation experiments were performed with dissected human AGM regions, yolk sacs, livers, umbilical cords and placentas from CS 12–17 human embryos (Figures 3.1.1 and 3.1.2). In total, 29 independent experiments were set up. Throughout the thesis, the term ‘independent’ refers to those experiments performed with different human embryos. The AGM region was obtained in all cases. The yolk sac, liver, umbilical cord and placenta were obtained in 20, 27, 17 and 19 experiments, respectively (Table 3.1.1).

HSCs in the AGM region were detected in 11 cases. In nine of these experiments, the AGM region was the only tissue containing HSCs, suggesting that the AGM region is the first generator of human HSCs. True multipotent HSCs in the human AGM region were detected from CS 14 until at least CS 17. Out of seven independent transplantations performed with CS 14 AGM regions, only one resulted in human

Figure 3.1.1. Representative human embryos used in the present study to establish the onset of HSC activity during human embryogenesis. Embryos at CS 12–17 were obtained and dissected immediately after elective termination of pregnancy. Scale bar, 10 mm.

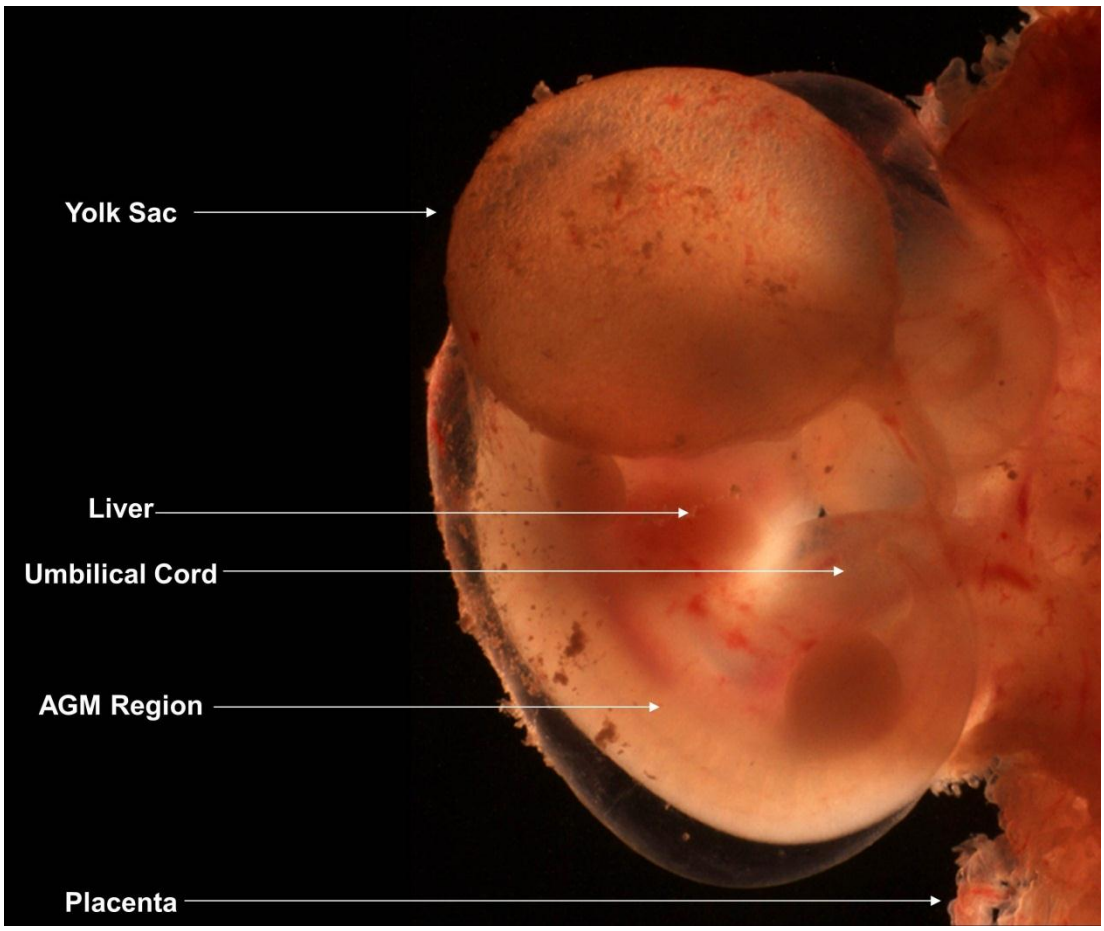


Figure 3.1.2. Embryonic tissues used in the present study to establish the spatial distribution of HSCs in the human embryo. In the majority of experiments, it was possible to dissect from the same human embryo all tissues that are considered to be involved in the embryonic development of the mammalian haematopoietic system.

Carnegie stage (Days*)	Embryonic tissues transplanted (Specimens with HSCs/All specimens)				
	AGM region	Yolk sac	Liver	Umbilical cord	Placenta
12 (26)	0/2	0/2	0/2	NA	1/2
13 (28)	0/2	NA	0/1	NA	1/2
14 (32)	1/7	0/7	0/7	0/4	2/7
15 (33)	4/5	0/3	0/5	0/4	1/2
16 (37)	2+1/6	1/4	0/6	0/6	1/3
17 (41)	4/7	2/4	1+2/6	0/3	1/3
Total	11+1/29	3/20	1+2/27	0/17	7/19

*Post-ovulatory gestational age as described by O'Rahilly and Müller (1987)
Long-term embryonic multiineage hematopoietic repopulation (HSCs)
Long-term embryonic unilineage T cell repopulation
Transient maternal unilineage T cell repopulation

Table 3.1.1. Spatio-temporal distribution of HSCs in the early human embryo. Cell suspensions prepared from AGM regions, yolk sacs, livers, umbilical cords and placentas obtained from CS 12–17 human embryos were individually transplanted into sublethally irradiated NSG mice. Data shown are the number of tissues that gave human haematopoietic repopulation (long-term embryonic multilineage repopulation, long-term embryonic unilineage T cell repopulation or transient maternal unilineage T cell repopulation) compared to the total number of tissues transplanted. The numbers in parentheses indicate an approximate postovulatory gestational age in days accepted for each CS (O'Rahilly and Muller, 1987). NA, not assessed.

long-term multilineage repopulation of a recipient mouse. In contrast, every second CS 15–17 human embryos contained HSCs in the AGM region. It was not possible to establish when the human AGM region loses HSC activity since human embryos beyond CS 17 could not be obtained for the present study (Table 3.1.1).

In the yolk sac, HSCs could be detected starting from CS 16. Thus, HSCs in the human yolk sac appear approximately 5 days later than in the AGM region. In total, there were observed only three cases when HSCs resided in the yolk sac. In two of these experiments, HSCs were also detected in the AGM region (Table 3.1.1). It is currently unclear whether the human yolk sac is able to produce HSCs independently or serves just as a reservoir for the HSCs produced in the AGM region and delivered to the yolk sac through the circulation.

In one out of 27 experiments, HSCs were found in the human embryonic liver. The liver was obtained from a CS 17 human embryo. In this embryo, HSCs were also found in the AGM region. Additionally, some cells capable of long-term unilineage T cell repopulation were detected in two other CS 17 livers. The same outcome was also observed in one experiment with the AGM region dissected from a CS 16 human embryo (Table 3.1.1). Importantly, the bone marrow from the NSG recipient mice exhibiting long-term unilineage T cell engraftment could never provide human haematopoietic repopulation in secondary recipients, excluding HSC origin of the repopulation. It can be speculated that cells capable of long-term unilineage T cell engraftment were early embryonic T cell progenitors. Alternatively, these cells might be human counterparts of mouse lymphopoiesis-biased γ and δ subtypes of long-term

repopulating haematopoietic cells found in the mouse adult bone marrow and the foetal liver (Dykstra *et al.*, 2007b).

Umbilical cords and placentas obtained from early human embryos contained no HSCs at all embryonic developmental stages tested. However, upon transplantation into sublethally irradiated NSG recipient mice, every third placenta irrespective of developmental stage could easily provide transient unilineage T cell repopulation of maternal origin (Table 3.1.1). Explicit details on the nature of this haematopoietic repopulation are provided in section 3.1.10.

3.1.4. Dorsal Aorta Is the Source of HSC Activity within the Human AGM Region

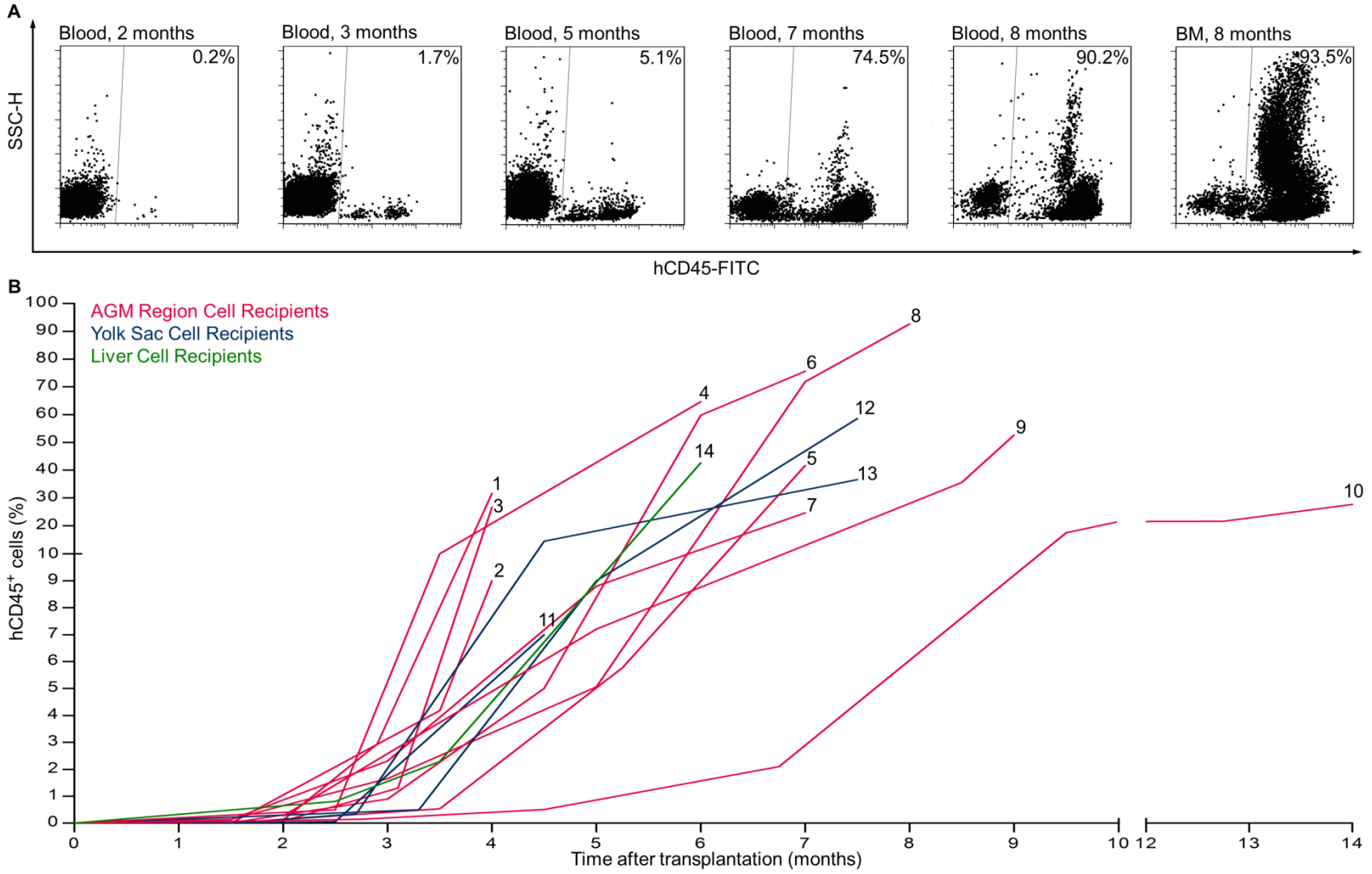
In the mouse embryo, definitive HSCs first appear in the dorsal aorta (de Bruijn *et al.*, 2000; Taoudi and Medvinsky, 2007). In 10 independent transplantation experiments, it was tested whether this could be extrapolated to the human embryo. To increase the success rate of these experiments, CS 15–17 human embryos were used. In each case, the AGM region was subdissected into the dorsal aorta and the UGRs, as previously described for the mouse AGM region (de Bruijn *et al.*, 2000; Taoudi and Medvinsky, 2007). Single cell suspensions prepared from these tissues were individually transplanted into sublethally irradiated NSG recipient mice. In 10 independent experiments performed, HSCs were detected in six dorsal aortae and never in the UGRs (data not shown).

3.1.5. High-Level Haematopoietic Repopulation by Early Human Embryonic HSCs

In NSG mice engrafted with HSCs from the human AGM region, the yolk sac and the embryonic liver, donor $CD45^+$ haematopoietic cells could first be detected in the recipient blood around month 2 after transplantation, reaching on average 0.22±0.15% of total blood leukocytes (Figure 3.1.3). Observing the recipient mice for up to 14 months after transplantation, it has been found that around month 5 after transplantation a substantial increase in the levels of donor haematopoietic contribution into the recipient peripheral blood usually occurs (Figure 3.1.3). The percentage of human $CD45^+$ cells in the recipient mouse peripheral blood could reach up to 90% of total leukocytes by month 8 after transplantation (Figure 3.1.3 and Table 3.1.2). By the end of the observation period, which lasted for 4–14 months after transplantation, on average 40±25% of total recipient blood leukocytes were of human origin. In the recipient mouse bone marrow and spleen, the percentage of human $CD45^+$ cells was even higher than in the peripheral blood, reaching on average 76±20% and 70±29% of total bone marrow or spleen $CD45^+$ cells, respectively (Table 3.1.2). In the recipient thymus, virtually all leukocytes were human T cells.

3.1.6. Multilineage Differentiation Potential of Early Human Embryonic HSCs

In general, the pattern of human multilineage haematopoietic repopulation observed upon the engraftment of early human embryonic HSCs was similar to that reported by others after engraftment of umbilical cord blood or adult bone marrow HSCs (Ishikawa *et al.*, 2005; Shultz *et al.*, 2005). As expected, haematopoietic


A
Blood, 2 months
0.2%
Blood, 3 months
1.7%
Blood, 5 months
5.1%
Blood, 7 months
74.5%
Blood, 8 months
90.2%
BM, 8 months
93.5%
SSC-H
hCD45-FITC
B
AGM Region Cell Recipients
Yolk Sac Cell Recipients
Liver Cell Recipients
hCD45+ cells (%)
Time after transplantation (months)

Recipient no.	Tissues transplanted	Time after transplantation, months	Human CD45+ cells in the recipient blood, %	Human CD45+ cells in the recipient bone marrow, %	Human CD45+ cells in the recipient spleen, %
1	AGM region	4	29	42	4
2	AGM region	4	9	NA	NA
3	AGM region	4	24	45	31
4	AGM region	6	62	92	74
5	AGM region	7	39	86	87
6	AGM region	7	73	93	93
7	AGM region	7	22	71	75
8	AGM region	8	90	94	96
9	AGM region	9	50	96	78
10	AGM region	14	25	60	70
11	Yolk sac	4.5	7	NA	NA
12	Yolk sac	7.5	56	NA	NA
13	Yolk sac	7.5	34	79	87
14	Liver	6	40	94	51

Table 3.1.2. High-level hematopoietic repopulation by early human embryonic HSCs. NSG mice transplanted with HSCs derived from the human AGM region, the yolk sac or the embryonic liver were observed for 4–14 months. At the end of each experiment, the percentage of human $CD45^+$ cells in the recipient peripheral blood, bone marrow and spleen was assessed by flow cytometry. NA, not assessed as the recipients died.

Figure 3.1.3 (on the previous page). Progressive growth of haematopoietic contribution in NSG recipient mice engrafted with early human embryonic HSCs. (A) Representative flow cytometry plots show hematopoietic cells in the peripheral blood and bone marrow of an NSG mouse transplanted with 0.33 e.e. of AGM region cells. In this case, the AGM region was obtained from a CS 16 embryo. The repopulation kinetics was monitored for 8 months. BM, bone marrow. **(B)** Human hematopoietic engraftment kinetics in the peripheral blood of 14 NSG recipient mice transplanted with human AGM region, yolk sac or embryonic liver cells. The numbers at the end of the time series correspond to the recipient identification numbers in Table 3.1.2.

differentiation was usually biased towards lymphopoiesis. In NSG recipient mice engrafted with early human embryonic HSCs, human $CD19^+$ B lineage cells were the major human leukocyte population in the peripheral blood (43±27%) and spleen (59±25%). In the recipient mouse bone marrow the percentage of B lineage cells was on average 38±23% of total human leukocytes (Table 3.1.3). To characterise human B lymphopoiesis and to distinguish between pre-B cells and B cells, human B lineage cells were examined for the surface expression of IgM. In the bone marrow of NSG recipient mice, approximately one-third (27±1.5%) of human B lineage cells passed the pre-B cell stage, as indicated by the expression of IgM. In the spleen, the proportion of IgM^+ B lineage cells was approximately three times as high (83±6.5%) as in the bone marrow. In the peripheral blood, almost all (93±3.4%) human B lineage cells were IgM^+. (Figure 3.1.4 A) These observations correspond to the normal development of B lineage cells (Nagasawa, 2006).

As for T lymphopoiesis, human $CD3^+$ T cells were observed in all main haematopoietic tissues of NSG mice engrafted with early human embryonic HSCs (Table 3.1.3). In the recipient blood, bone marrow and spleen, the percentage of these cells was 35±20%, 14±7.8% and 26±21% of total human leukocytes, respectively. In the thymus, all $CD45^+$ haematopoietic cells were human T cells. The majority of them (77±10%) were double-positive $CD4^+CD8^+$ T cells. Thymic single-positive $CD4^+CD8^-$ and $CD8^+CD4^-$ T cells constituted 16±8.3% and 5.5±2.5% of total human T cells, respectively. As expected, only 2.1±0.64% of human thymic $CD45^+$ cells were double-negative $CD4^-CD8^-$ T cells. Importantly, only rare (0.41±0.18%) human leukocytes in the peripheral blood were double-positive T cells,

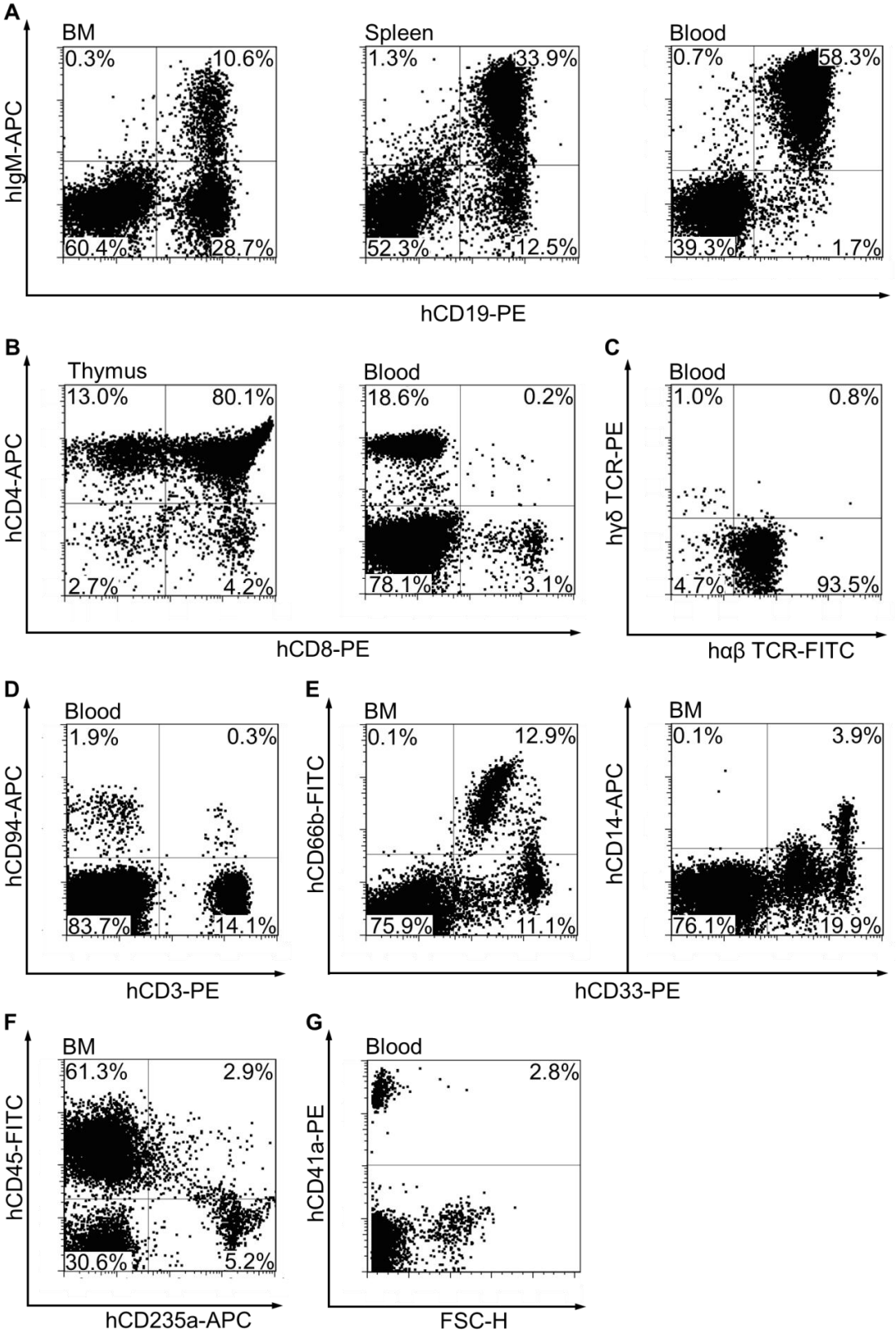

A
BM
0.3%
10.6%
60.4%
28.7%
Spleen
1.3%
33.9%
52.3%
12.5%
Blood
0.7%
58.3%
39.3%
1.7%
hIgM-APC
hCD19-PE
B
Thymus
13.0%
80.1%
2.7%
4.2%
Blood
18.6%
0.2%
78.1%
3.1%
hCD4-APC
hCD8-PE
C
Blood
1.0%
0.8%
4.7%
93.5%
hγδ TCR-PE
hαβ TCR-FITC
D
Blood
1.9%
0.3%
83.7%
14.1%
hCD94-APC
hCD3-PE
E
BM
0.1%
12.9%
75.9%
11.1%
hCD66b-FITC
BM
0.1%
3.9%
76.1%
19.9%
hCD14-APC
hCD33-PE
F
BM
61.3%
2.9%
30.6%
5.2%
hCD45-FITC
hCD235a-APC
G
Blood
2.8%
hCD41a-PE
FSC-H

Figure 3.1.4 (on the previous page). Human long-term multilineage haematopoietic repopulation in NSG recipient mice engrafted with early human embryonic HSCs. Representative flow cytometry plots show human B cells **(A)**, T cells **(B, C)**, NK and NKT cells **(D)**, granulocytes and monocytes/macrophages **(E)**, erythroid cells **(F)** and platelets **(G)** in the peripheral blood, bone marrow, spleen and/or thymus of an NSG mouse 7 months after it was transplanted with 0.5 e.e. of AGM region cells. In this case, the AGM region was obtained from a CS 17 human embryo. Note that the staining for CD4 and CD8 is shown in gated human $CD45^+$ cells, and for TCRs in gated human $CD3^+$ cells. For flow cytometry controls see Figure A.1. BM, bone marrow.

Recipient no.	Blood				Bone marrow				Spleen				Thymus			
	B	T	NK	Myeloid	B	T	NK	Myeloid	B	T	NK	Myeloid	$CD4^-CD8^-$	$CD4^+CD8^+$	$CD4^+CD8^-$	$CD8^+CD4^-$
	% in the human $CD45^+$ cell population															
1	44	39	NA	11	48	19	NA	30	78	12	NA	9	NA	NA	NA	NA
2	67	23	3	4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
3	19	65	5	11	16	42	1	38	29	56	1	15	1	68	25	6
4	73	12	3	12	51	2	0.62	46	86	4	1.5	8	2	80	11	7
5	77	5	4	12	51	0.05	0.16	45	87	1	1	9	2	84	10	4
6	75	19	2	4	62	1	0.25	37	90	7	0.88	2	3	80	13	4
7	56	25	9	9	61	19	1	20	82	4	3	14	2	85	9	4
8	17	36	31	18	20	4	1.5	74	41	39	11	11	NA	NA	NA	NA
9	1.4	63	10	26	2.2	2.7	1	95	28	34	4	20	NA	NA	NA	NA
10	7	61	5	31	6	9	2	83	35	54	2	10	2	59	29	10
11	59	22	NA	15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
12	63	19	NA	14	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
13	26	60	9	6	42	12	2	43	46	41	7	7	2	69	22	7
14	24	40	20	19	58	10	4	28	45	35	6	14	3	88	7	2

Table 3.1.3. Multilineage haematopoietic engraftment in primary recipient mice. Data shown are the percentage of B cells ($CD19^+$), T cells ($CD3^+$ or $CD4^+CD8^+$, $CD4^+CD8^-$ and $CD8^+CD4^-$), NK cells ($CD94^+CD3^-$) and myeloid cells ($CD33^+$ or $CD13^+$) in the human $CD45^+$ cell population in the peripheral blood, bone marrow, spleen and thymus of NSG recipient mice transplanted with human AGM region, yolk sac or embryonic liver cells. The recipient identification numbers are the same as in Table 3.1.2. NA, not assessed.

as is normally observed in human peripheral blood (Figure 3.1.4 B and Table 3.1.3). The above indicates that early human embryonic HSC-derived T cell progenitors are capable of normal intrathymic maturation (reviewed by Koch and Radtke, 2011). Additionally, 97±1.5% of human $CD3^+$ cells in the peripheral blood of NSG recipient mice expressed αβ T cell receptors. Only 1.0±0.75% of T cells expressed γδ T cell receptors (Figure 3.1.4 C). The same is normally observed in the human peripheral blood (reviewed by Ciofani and Zuniga-Pflucker, 2010).

To finalise the description of lymphopoiesis, it needs to be mentioned that early human embryonic HSC were also capable of producing $CD94^+CD3^-$ NK cells and $CD94^+CD3^+$ NKT cells. These cells were present at a comparatively low frequency in the recipient NSG mouse peripheral blood (9.2±8.9%), bone marrow (1.4±1.1%) and spleen (3.7±3.4%) (Figure 3.1.4 D).

As for myelopoiesis, the percentage of human $CD33^+$ myeloid cells in the peripheral blood and spleen of NSG recipient mice engrafted with early human HSCs was comparatively low (14±7.8%). However, up to 95% of human leukocytes in the recipient bone marrow were of myeloid lineage. On average, this figure was 49±24% (Table 3.1.3). Both human $CD33^+CD66b^+$ granulocytes and $CD33^+CD14^+$ macrophages were observed (Figure 3.1.4 E). Human erythroid cells could never be detected in the peripheral blood and spleen of NSG mice engrafted with early human embryonic HSCs. However, human $CD235a^+$ erythroid cells could easily be found in the recipient mouse bone marrow, reaching 6.2±3.2% of total bone marrow nucleated cells. On average, 18±7.2% of erythroid cells coexpressed CD45, indicating their

immature state (Figure 3.1.4 F). Mouse erythropoietin has a reduced activity on human cells (Nicolini *et al.*, 1999). This may be one of the reasons for the lack of peripheralisation of human erythroid cells. Also, human erythrocyte release into the mouse peripheral blood may be hampered by recipient macrophages (Hu *et al.*, 2011). As for megakaryocytopoiesis, human $FSC^{lo}CD41^{+}$ platelets were easily detectable in the peripheral blood of NSG mice engrafted with early human embryonic HSCs (Figure 3.1.4 G).

3.1.7. Human HSCs and Haematopoietic Progenitors in the Recipient Mouse Bone Marrow

Flow cytometry analysis of the bone marrow from NSG recipient mice engrafted with HSCs from the human AGM region, the yolk sac and the embryonic liver have confirmed that human haematopoiesis in such recipients progressed from HSCs to mature haematopoietic cells through intermediate haematopoietic progenitor stages. In the human bone marrow and umbilical cord blood, the $CD34^{+}CD38^{-}$ cell population contains the vast majority, if not all, of HSCs and haematopoietic progenitors (Bhatia *et al.*, 1997; Doulatov *et al.*, 2010). The frequency of human $CD34^{+}CD38^{-}$ cells in the bone marrow of recipient mice transplanted with early human embryonic HSCs was on average 1.0±0.71% of total bone marrow nucleated cells (Table 3.1.4), a value approximately 10 times higher than is usually observed in the normal human bone marrow (Pang *et al.*, 2011). For recipient mice 4, 9, 10 and 14, a more detailed flow cytometry analysis of the bone marrow human HSC and haematopoietic progenitor compartment was performed. The human $CD34^{+}CD38^{-}$ cell population was further subdivided into three cell subpopulations based on the

Recipient no.	Human $CD34^+CD38^-$ cells in the recipient bone marrow, %
1	NA
2	NA
3	0.37
4	1.1
5	2.5
6	1.2
7	1.9
8	1.1
9	0.41
10	0.29
11	NA
12	NA
13	0.66
14	0.78

Table 3.1.4. Human $CD34^+CD38^-$ cell frequency in the bone marrow of NSG recipient mice engrafted with early human embryonic HSCs. NSG mice transplanted with HSCs derived from the human AGM region, the yolk sac or the embryonic liver were observed for 4–14 months. At the end of each experiment, the percentage of human $CD34^+CD38^-$ cells in the recipient bone was assessed by flow cytometry. The recipient identification numbers are the same as in Table 3.1.2. NA, not assessed.

expression of Thy-1 and CD45RA (Figure 3.1.5). Human immunophenotypic HSCs ($CD34^+CD38^-Thy\text{-}1^+CD45RA^-$), MPPs ($CD34^+CD38^-Thy\text{-}1^-CD45RA^-$) and MLPs ($CD34^+CD38^-Thy\text{-}1^-CD45RA^+$) could easily be identified in the recipient bone marrow, constituting on average 25±6.5%, 22±12% and 54±14% of human $CD34^+CD38^-$ cells, respectively. If we compare these figures to those for the normal human bone marrow (Majeti *et al.*, 2007), MLPs but not MPPs were dominant haematopoietic progenitors in the NSG recipient mouse bone marrow.

To assess human haematopoietic progenitors in the recipient mouse bone marrow functionally, the CFU-C assay was performed. Bone marrow cells from the recipient mice engrafted with early human embryonic HSCs were plated into methylcellulose medium supplemented with human cytokines. After 14 days of culture, human haematopoietic colonies could easily be detected (Figure 3.1.6 A–D). In the recipient mouse bone marrow, there were on average 31.8±10.3 human CFU-Cs per 10000 nucleated cells (Table 3.1.5). The human origin of haematopoietic colonies was confirmed by flow cytometry analysis (Figure 3.1.6 E and F).

3.1.8. Spatial Distribution of Haematopoietic Cells in the Recipient Mouse Bone Marrow

In two experiments, femurs were collected from NSG recipient mice engrafted with HSCs from the human AGM region. The femurs were fixed, decalcified, snap-frozen in OCT embedding medium and sectioned. After staining with anti-human and anti-mouse CD45 monoclonal antibodies, the spatial organisation of human and mouse haematopoietic cells in the recipient bone marrow was assessed using confocal

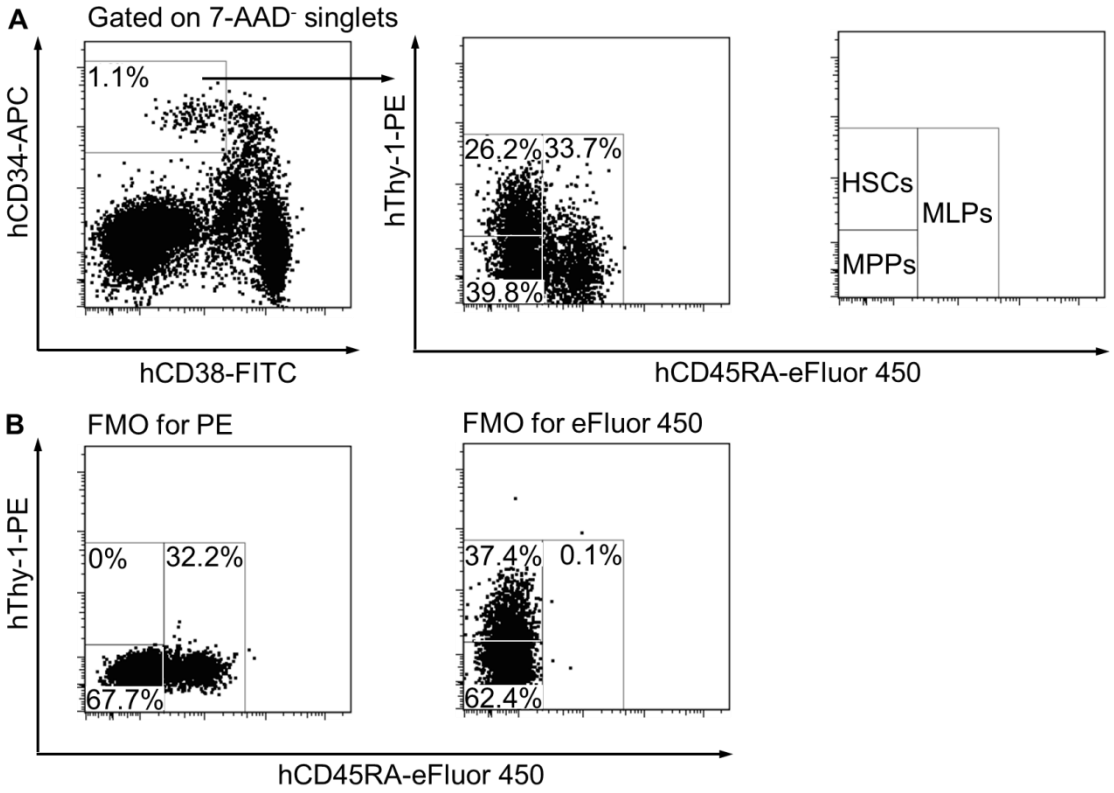


Figure 3.1.5. Human immunophenotypic HSCs, MPPs and MLPs in the bone marrow of NSG recipient mice engrafted with early human embryonic HSCs. Representative flow cytometry plots show the flow cytometry analysis performed with bone marrow cells from recipient mouse 4 engrafted with AGM region HSCs from a CS 17 human embryo. To distinguish between human immunophenotypic HSCs ($CD34^{+}CD38^{-}Thy\text{-}1^{+}CD45RA^{-}$), MPPs ($CD34^{+}CD38^{-}Thy\text{-}1^{-}CD45RA^{-}$) and MLPs ($CD34^{+}CD38^{-}Thy\text{-}1^{-}CD45RA^{+}$), the cells were immunolabelled with anti-human CD34, CD38, Thy-1 and CD45RA monoclonal antibodies and analysed by flow cytometry **(A)**. Cell populations of interest were gated based on fluorescence minus one (FMO) controls **(B)**.

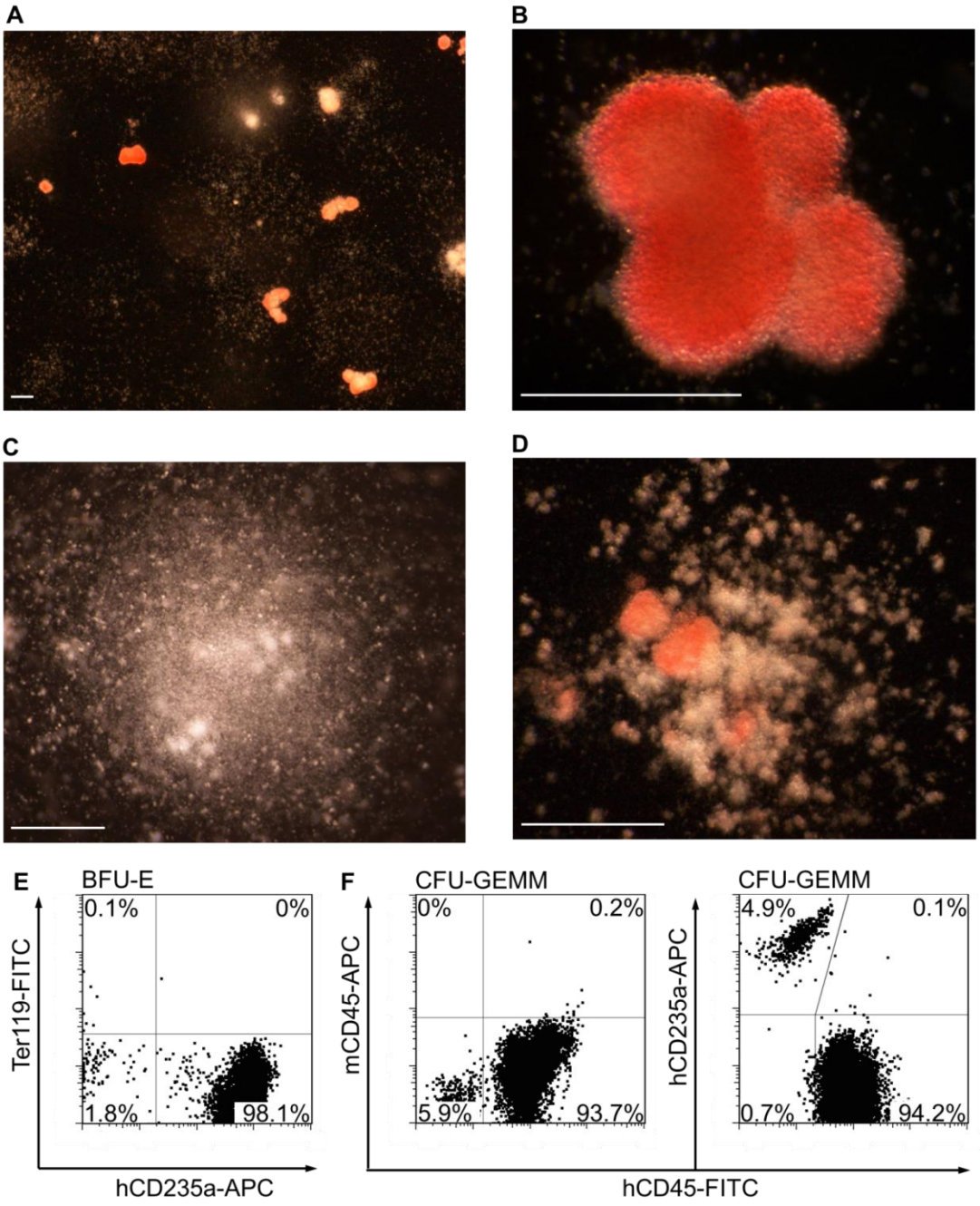


Figure 3.1.6. Assessment of human CFU-C activity in the bone marrow of NSG recipient mice engrafted with early human embryonic HSCs. Seven months after transplantation, bone marrow cells from an NSG mouse repopulated with AGM region HSCs from a CS 17 human embryo were plated into methylcellulose medium supplemented with human cytokines. This resulted in the formation of human haematopoietic colonies of different types **(A–D)**. Representative flow cytometry plots confirm the human origin of haematopoietic colonies generated **(E, F)**. Scale bars, 0.5 mm.

Recipient no.	Human CFU-C no. per 10000 input bone marrow nucleated cells		
	BFU-E	CFU-GM/G/M	CFU-GEMM
1	2.3±0.6	13.0±3.8	2.5±0.4
2	NA	NA	NA
3	8.7±1.5	34.7±11.2	1.7±0.6
4	NA	NA	NA
5	7.6±1.4	26.1±4.0	1.7±0.2
6	5.0±1.4	31.0±1.4	0.5±0.7
7	6.3±0.1	16.3±0.4	1.6±0.3
8	6.5±0.7	33.0±4.2	1.5±0.7
9	NA	NA	NA
10	NA	NA	NA
11	NA	NA	NA
12	NA	NA	NA
13	5.6±0.6	15.4±2.0	1.8±0.3
14	NA	NA	NA

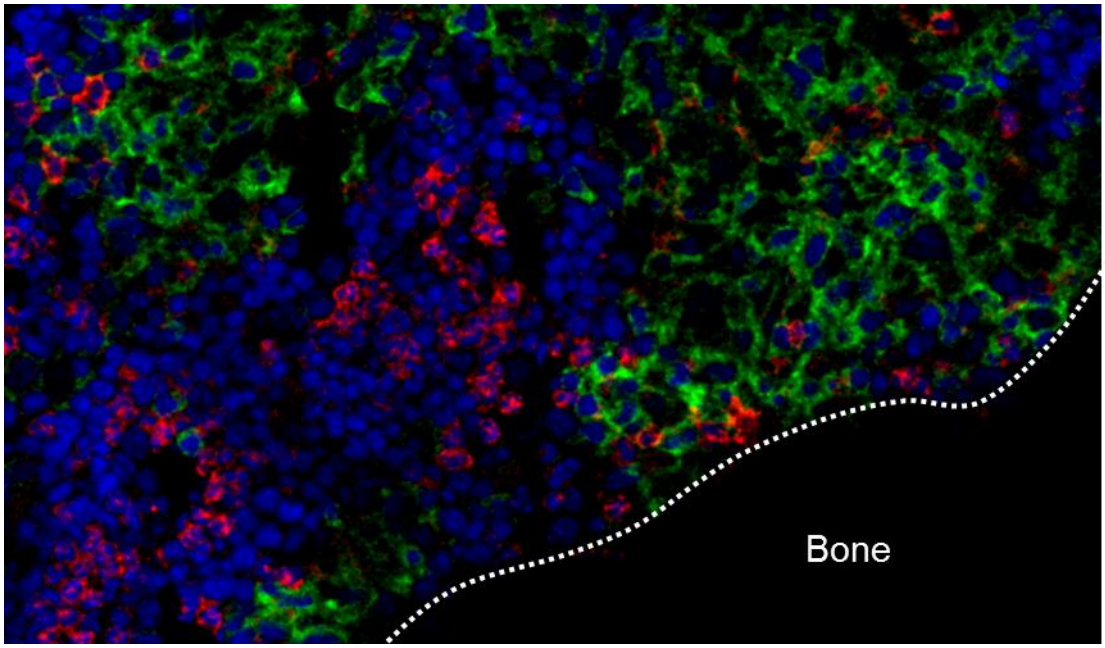
Table 3.1.5. Human CFU-C frequency in the bone marrow of NSG recipient mice engrafted with early human embryonic HSCs. Data shown are the number±SD of human CFU-Cs per 10000 input bone marrow nucleated cells. The bone marrow was obtained from NSG mice repopulated with HSCs from the human AGM region, the yolk sac or the embryonic liver. The recipient identification numbers are the same as in Table 3.1.2. NA, not assessed.

microscopy. In recipient mouse 3, in which 45% of bone marrow $CD45^+$ cells were of human origin, human and mouse haematopoietic cells in the bone marrow were segregated and formed well-demarcated areas (Figure 3.1.7 A). Only between these areas, human and mouse haematopoietic cells have been found intermixed. In the recipient mouse 6, in which 93% of total bone marrow $CD45^+$ cells were of human origin, human haematopoietic cells almost completely superseded mouse haematopoietic cells in the bone marrow (Figure 3.1.7 B). The above observations suggest that upon the xenogeneic transplantation of HSCs from the human AGM region, discrete areas of human and mouse haematopoiesis persist in the recipient mouse bone marrow. It would be of interest to test if this is also the case in human umbilical cord blood and adult bone marrow HSC xenotransplantation.

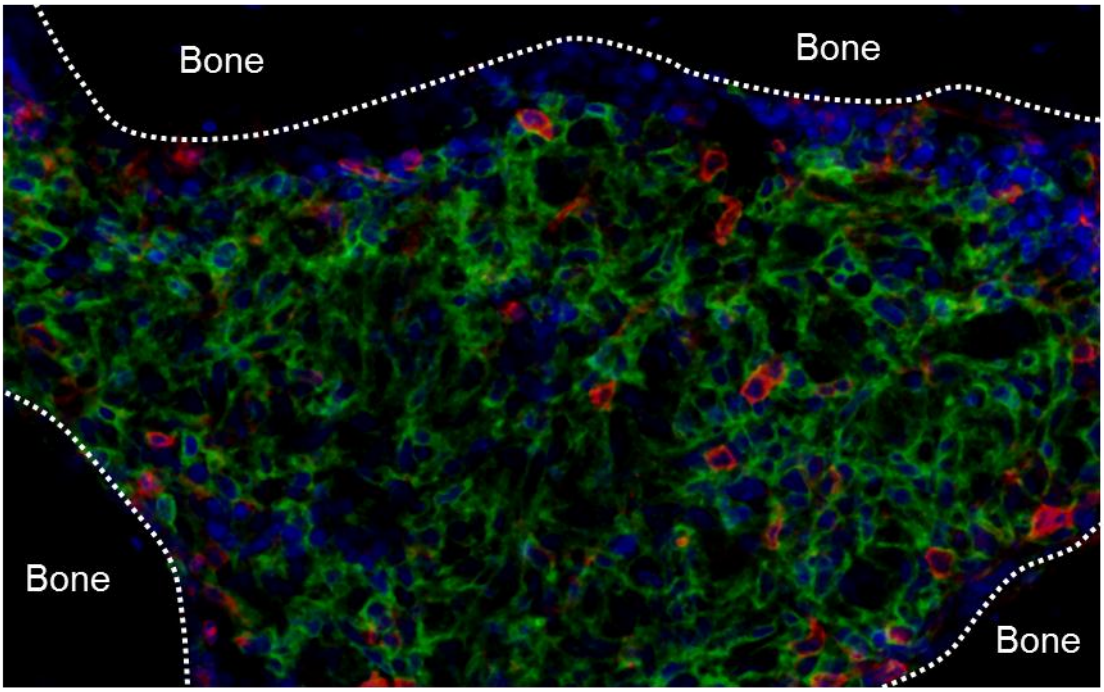
3.1.9. Early Human Embryonic HSCs Self-Renew and Are Retransplantable

It was tested whether HSCs from the human AGM region, the yolk sac and the embryonic liver can be serially transplanted. In all eleven experiments with the AGM region, two experiments with the yolk sac and one experiment with the embryonic liver, the transplantation of the bone marrow from one primary recipient mouse into six to 20 secondary recipient mice resulted in sustainable human long-term multilineage haematopoietic engraftment (Figure 3.1.8). In these experiments, 96% (108 out of 113) of secondary recipient mice transplanted were found engrafted with human HSCs. Since the vast majority of primary recipient mice were transplanted with approximately one or two early human embryonic HSCs (see section 3.2.3), these observations suggest that early human embryonic HSCs self-renewed in the

A



B



hCD45 FITC

mCD45 Alexa Fluor 546

DAPI

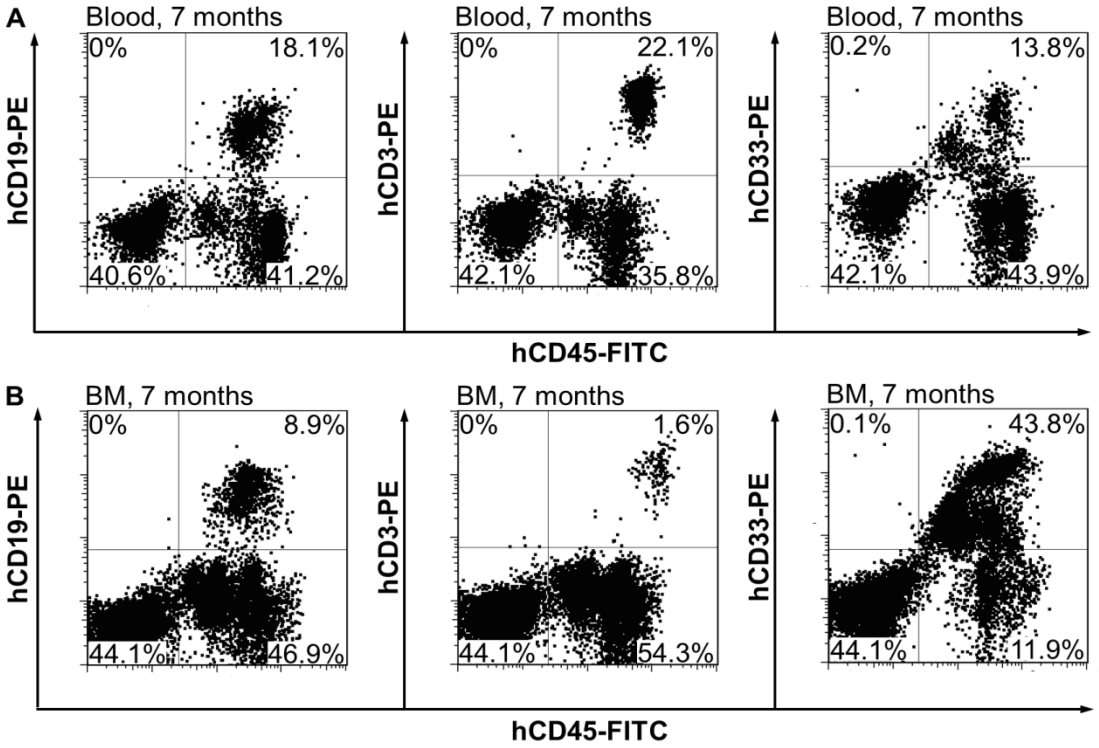


Figure 3.1.8. Human long-term multilineage haematopoietic engraftment in secondary NSG recipient mice upon the retransplantation of early human embryonic HSCs. Representative flow cytometry plots show human B cells ($CD45^+CD19^+$), T cells ($CD45^+CD3^+$) and myeloid cells ($CD45^+CD33^+$) in the peripheral blood **(A)** and the bone marrow **(B)** of an NSG mouse transplanted with bone marrow cells from a primary recipient engrafted with HSCs from a CS 15 AGM region. Secondary transplantation was performed 7 months after primary transplantation. The analysis of secondary recipient mice was performed 7 months later. In total, 14 independent secondary transplantation experiments were performed.

Figure 3.1.7 (on the previous page). Human haematopoietic areas in the bone marrow of NSG recipient mice engrafted with HSCs from the human AGM region. Femurs were obtained from two NSG recipient mice exhibiting human long-term multilineage haematopoietic repopulation upon the transplantation with HSCs from the human AGM region. Recipient mice 3 **(A)** and 6 **(B)** were used in this experiment (see Tables 3.1.2, 3.1.3 and 3.1.4 for the details on these recipients). After the bones were fixed, decalcified, snap-frozen and sectioned, tissues were stained with anti-human and anti-mouse CD45 monoclonal antibodies, followed by nuclear counterstaining with DAPI. Images were obtained employing confocal microscopy. A control for anti-human CD45 monoclonal antibody staining is shown on Figure B.1.

bone marrow of primary recipient mice, which is discussed in more detail in the next result subchapter.

3.1.10. HSCs in the Human Placenta Appear Later than in the AGM Region

It has recently been reported that the human placenta harbours HSCs from at least week 6 of gestation (Robin *et al.*, 2009). In that study, 17 human placentas from human embryos and foetuses between weeks 6 and 19 of development were screened for HSC activity by transplantation into sublethally irradiated NOD/SCID mice. It has been reported that human haematopoietic repopulation of recipient mice was achieved at least in five independent experiments. However, among these five cases there were only one placenta from a 6-week-old human embryo and one placenta from an 8-week-old human embryo. Since Robin *et al.* (2009) used postmenstrual gestational age, which exceeds by approximately 2 weeks postovulatory gestational age employed in the Carnegie staging system, 6–8-week-old human embryos in their study are comparable to CS 12–17 human embryos in the present study. Juxtaposing the data of Robin *et al.* (2009) with the data on the timing of HSC emergence in the human AGM region presented in section 3.1.3, one can conclude that in the early human embryo HSCs appear in the placenta simultaneously with or even earlier than in the AGM region.

To validate the above claims regarding the presence of HSCs in the early human placenta, 19 independent transplantation experiments were performed with cells obtained from CS 12–17 human placentas (Table 3.1.1). Prior to transplantation, in six experiments, placental tissues were subject to enzymatic digestion and Ficoll

density gradient separation as described by Robin *et al.* (2009) or Barcena *et al.* (2009). In 13 cases, Ficoll density gradient separation step was omitted to minimise cell losses. Despite this and the fact that in the present study placental cells were transplanted into NSG mice, which are more receptive for human HSCs than NOD/SCID mice (McDermott *et al.*, 2010), in none of the experiments human long-term multilineage haematopoietic engraftment could be achieved. However, in seven experiments, 12 out of 22 mice transplanted with human placental cells were found repopulated exclusively with human T cells and showed signs of graft-versus-host disease (Table 3.1.1). If the recipient mice did not develop graft-versus-host disease, T cells could hardly be detected in recipient haematopoietic tissues at month 4–5 after transplantation (Figure 3.1.9). If the recipient mice developed graft-versus-host disease, human T cells propagated and in some cases constituted virtually 100% of recipient blood leukocytes by the time of animal death.

It has previously been reported that T cells from human blood expand easily after transfer into immunodeficient mice (van Rijn et al., 2003), and placenta is always contaminated with maternal blood. To discriminate between maternal versus embryonic origin of human T cells in NSG recipient mice transplanted with human placental cells, DNA fingerprinting was carried out employing STR analysis. Genomic DNA was extracted from human embryonic donor tissues and peripheral blood, bone marrow and/or spleen cells from corresponding recipient mice. In all cases tested, human cells in the recipient mice exhibiting transient human unilineage T cell repopulation following the transplantation of placental cells were of maternal origin (Table 3.1.6). In contrast, human cells in the recipient mice found repopulated

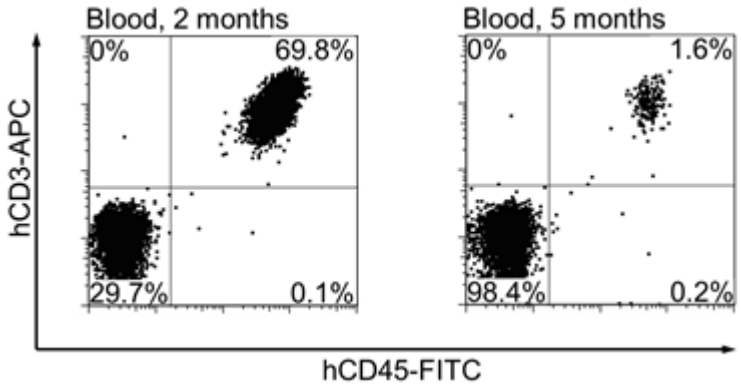


Figure 3.1.9. Human transient unilineage T cell repopulation in NSG recipient mice transplanted with cells from the early human placenta. Representative flow cytometry plots show human T cells ($CD45^+CD3^+$) in the peripheral blood of a recipient mouse transplanted with 0.33 e.e of a CS 16 placenta. Peripheral blood leukocytes from this recipient were analysed 2 and 5 months after transplantation.

Tables 3.1.6–3.1.11 (on the following six pages). Results of the STR analysis performed to distinguish embryonic versus maternal origin of haematopoietic repopulation in NSG recipient mice transplanted with human embryo or placenta-derived cells. STR profiles (the array of alleles in multiple STR loci) were obtained for genomic DNA extracted from embryonic/foetal tissues and from long-term hematopoietic graft (peripheral blood, spleen and/or bone marrow cells from repopulated NSG recipient mice). Numbers denote the alleles in a given STR locus. When identical STR profiles were obtained for embryonic/foetal and graft samples, this confirmed the embryonic/foetal origin of haematopoietic graft. By contrast, in the case with CS 12–17 placentas (Table 3.1.6), the match of only one allele in the majority of STR loci for embryonic and graft samples indicated familial relationship only, thus demonstrating the maternal origin of engrafted cells. Amelogenin X and Y PCR products were used for gender determination. F, allele drop-out. Pm, match probability (the probability that a human genomic DNA sample that is chosen at random has a matching STR profile to the embryonic/foetal sample).

Table 3.1.6. Early placenta, transient unilineage T cell repopulation

Locus	Embryo 1	Graft 1	Embryo 2	Graft 2	Embryo 3	Graft 3
D3S1358	16, 16	16, 17	17, 17	17, 18	14, 16	16, 18
vWA	16, 17	14, 17	14, 17	15, 17	16, 19	18, 19
D16S539	11, 12	11, 12	9, 12	9, 11	11, 12	12, 12
D2S1338	23, 25	17, 23	24, 27	16, 27	17, 19	21, F
D8S1179	12, 12	11, 12	13, 15	8, 15	13, 13	13, 13
D21S11	31.2, 32.2	31, 31.2	30, 33.2	28, 33.2	29, 31	31, 31
D18S51	13, 16	12, 13	13, 16	13, 13	12, 15	14, 15
D19S433	13, 14	13, 14	12, 14	12, 13	12, 15	12, F
TH01	6, 9.3	6, 9.3	7, 7	7, 9.3	6, 9.3	9.3, F
FGA	19, 21	19, 20	21, 24	21, 24	25, 25	23, 25
Amelogenin	XY	XX	XY	XX	XY	XX
Result	No match, familial relationship		No match, familial relationship		No match, familial relationship	

Table 3.1.7. AGM region, long-term multilineage haematopoietic repopulation

Locus	Embryo 1	Graft 1	Embryo 2	Graft 2	Embryo 3	Graft 3
D3S1358	16, 16	16, 16	17, 17	17, 17	16, 16	16, 16
vWA	16, 17	16, 17	14, 17	14, 17	15, 16	15, 16
D16S539	11, 12	11, 12	8, 11	8, 11	9, 11	9, 11
D2S1338	23, 25	23, 25	20, 20	20, 20	20, 20	20, 20
D8S1179	9, 12	9, 12	13, 13	13, 13	8, 13	8, 13
D21S11	31.2, 33.2	31.2, 33.2	29, 30.2	29, 30.2	30, 30	30, 30
D18S51	13, 16	13, 16	12, 14	12, 14	12, 14	12, 14
D19S433	13, 14	13, 14	12, 14	12, 14	13, 14	13, 14
TH01	6, 9.3	6, 9.3	6, 9.3	6, 9.3	9.3, 9.3	9.3, 9.3
FGA	19, 21	19, 21	20, 22	20, 22	21, 23	21, 23
Amelogenin	XY	XY	XY	XY	XX	XX
Result	Match ($Pm=3.87\times10^{-13}$)		Match ($Pm=8.32\times10^{-15}$)		Match ($Pm=1.69\times10^{-13}$)	

Table 3.1.8. Yolk sac, long-term multilineage haematopoietic repopulation

Locus	Embryo 1	Graft 1	Embryo 2	Graft 2	Embryo 3	Graft 3
D3S1358	16, 17	16, 17	17, 17	17, 17	14, 15	14, 15
vWA	14, 17	14, 17	14, 17	14, 17	17, 17	17, 17
D16S539	11, 12	11, 12	8, 11	8, 11	11, 11	11, 11
D2S1338	17, 24	17, 24	20, 20	20, 20	19, 25	19, 25
D8S1179	13, 16	13, 16	13, 13	13, 13	10, 10	10, 10
D21S11	28, 32.2	28, 32.2	29, 30.2	29, 30.2	30, 30	30, 30
D18S51	15, 17	15, 17	12, 14	12, 14	14, 18	14, 18
D19S433	13, 13	13, 13	12, 14	12, 14	14, 15	14, 15
TH01	6, 9	6, 9	6, 9.3	6, 9.3	9.3, 9.3	9.3, 9.3
FGA	21, 21	21, 21	20, 22	20, 22	23, 25	23, 25
Amelogenin	XY	XY	XY	XY	XY	XY
Result	Match ($Pm=8.72\times10^{-14}$)		Match ($Pm=8.32\times10^{-15}$)		Match ($Pm=1.58\times10^{-13}$)	

Table 3.1.9. Liver, long-term unilineage T cell repopulation

Locus	Embryo 1	Graft 1	Embryo 2	Graft 2
D3S1358	14, 15	14, 15	16, 16	16, 16
vWA	17, 17	17, 17	15, 16	15, 16
D16S539	11, 11	11, 11	9, 11	9, 11
D2S1338	19, 25	19, 25	20, 20	20, 20
D8S1179	10, 10	10, 10	8, 13	8, 13
D21S11	30, 30	30, 30	30, 30	30, 30
D18S51	14, 18	14, 18	12, 14	12, 14
D19S433	14, 15	14, 15	13, 14	13, 14
TH01	9.3, 9.3	9.3, 9.3	9.3, 9.3	9.3, 9.3
FGA	23, 25	23, 25	21, 23	21, 23
Amelogenin	XY	XY	XX	XX
Result	Match ($Pm=1.58\times10^{-13}$)		Match ($Pm=1.69\times10^{-13}$)	

Table 3.1.10. AGM region, long-term unilineage T cell repopulation

Locus	Embryo	Graft
D3S1358	16, 18	16, 18
vWA	16, 17	16, 17
D16S539	11, 14	11, 14
D2S1338	16, 24	16, 24
D8S1179	12, 13	12, 13
D21S11	30, 31	30, 31
D18S51	15, 18	15, 18
D19S433	13, 13	13, 13
TH01	7, 9.3	7, 9.3
FGA	24, 25	24, 25
Amelogenin	XY	XY
Result	Match ($Pm=2.05\times10^{-14}$)	

Table 3.1.11. Late placenta, long-term multilineage haematopoietic repopulation

Locus	Embryo	Graft
D3S1358	14, 16	14, 16
vWA	16, 18	16, 18
D16S539	11, 11	11, 11
D2S1338	18, 20	18, 20
D8S1179	13, 14	13, 14
D21S11	29, 31.2	29, 31.2
D18S51	15, 16	15, 16
D19S433	13, 14	13, 14
TH01	6, 9.3	9.3, F
FGA	20, 25	20, 25
Amelogenin	XY	XY
Result	Match ($Pm=2.12\times10^{-12}$)	

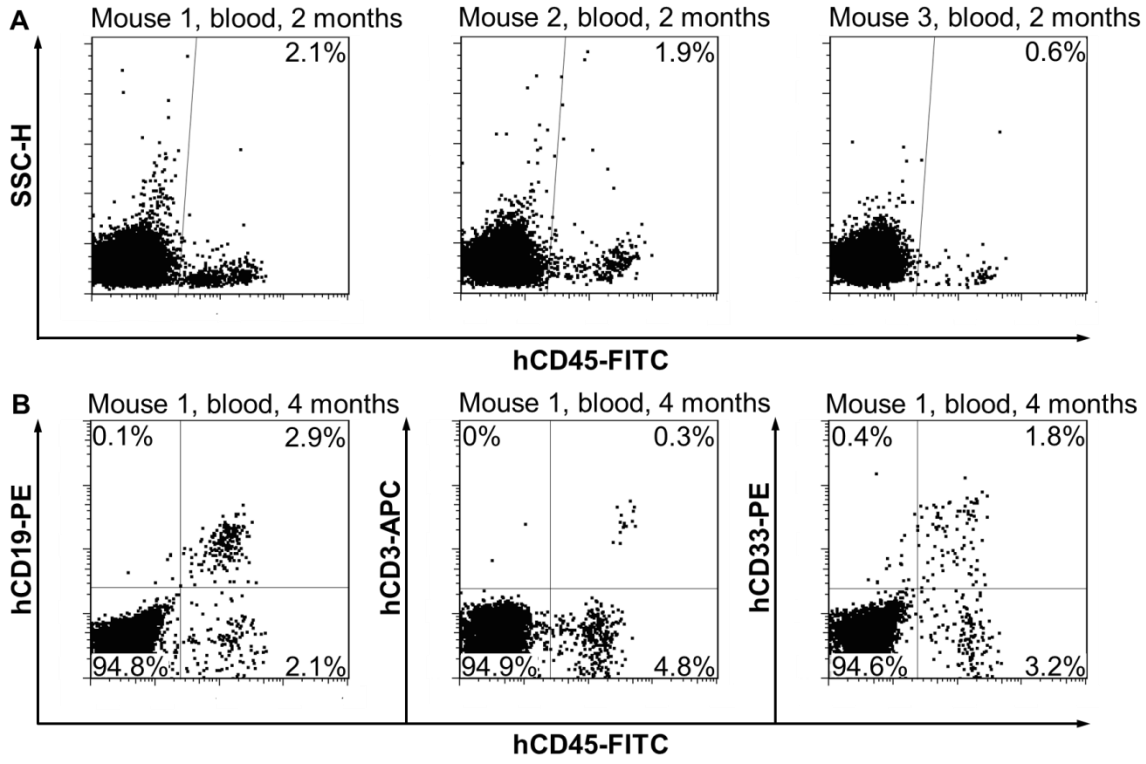


Figure 3.1.10. Human long-term multilineage haematopoietic repopulation in NSG recipient mice engrafted with HSCs from the midgestation human placenta. (A) Representative flow cytometry plots show the presence of human $CD45^+$ haematopoietic cells in the peripheral blood of three NSG recipient mice each transplanted with ~1/30 of total placental cells from a 15-week-old human foetus. Recipient peripheral blood was analyzed 2 months after transplantation. **(B)** Human B cells ($CD45^+CD19^+$), T cells ($CD45^+CD3^+$) and myeloid cells ($CD45^+CD33^+$) in the peripheral blood are shown for one of the recipients. The analysis was performed 4 months after transplantation. Two independent experiments were performed.

upon the transplantation of human AGM region, yolk sac or liver cells were always of embryonic origin (Tables 3.1.7–3.1.10).

To prove that the failure to detect HSCs in the early human placenta was not due to some technical problems in employing the protocol of Robin *et al.* (2009), placental tissues from two 15-week-old (postmentrual gestational age) human foetuses were obtained and processed exactly in the same way as it had been done for the early human placentas. In each of these two experiments, placental cells were transplanted into three sublethally irradiated NSG recipient mice at the dose of 1/30 of total placenta cells per recipient. All recipient mice achieved human long-term multilineage haematopoietic engraftment of foetal origin (Figure 3.1.10 and Table 3.1.11).

3.1.11. Postmenstrual Gestation Age Should Not Be Used in Studies on Early Human Embryos

To obtain consistent results in developmental biology studies, it is important to use internationally accepted embryo staging systems. Embryo staging systems exist for the zebrafish, frog, chicken, mouse, rat, rhesus macaque and some other animal embryos (Hamburger and Hamilton, 1951; Hendrickx and Sawyer, 1975; Kimmel *et al.*, 1995; Nieuwkoop and Faber, 1956; Theiler, 1989; Witschi, 1962). To stage human embryos, the Carnegie staging system has been introduced (O'Rahilly and Muller, 1987). Nevertheless, CSs are often substituted with postmenstrual gestational ages determined and provided by gynaecologists-obstetricians performing elective termination of pregnancy (Huyhn *et al.*, 1995; Migliaccio *et al.*, 1986; Oberlin *et al.*,

2010; Oberlin *et al.*, 2002; Robin *et al.*, 2009; Tavian *et al.*, 1996). Depending on the length of the woman's menstrual cycle, postmenstrual gestational age of the human embryo exceeds by 1–3 weeks its postovulatory gestational age, which is the real age of the embryo. Since the difference between the two gestational ages cannot be easily established, postmenstrual gestational age is an unreliable indicator of the developmental stage of the human embryo (O'Rahilly and Muller, 2000). In contrast, the Carnegie staging system, which was used in the present study, is based on a complex of both external and internal morphological criteria and is not directly linked to gestational age (O'Rahilly and Muller, 1987).

To determine the correlation between CSs and postmenstrual gestational age, information on a woman's menstrual history was collected for the vast majority (n=89) of human embryos obtained for the study. The human embryos were split into six groups based on the CS they belonged to. A mean postmenstrual gestational age and a 95% CI were calculated for each group. For CS 12–15, 95% CIs overlapped between the four groups, indicating that postmenstrual gestational age cannot be used to distinguish between CS 12–15 human embryos. However, based on postmenstrual gestational age, it was possible to distinguish between CS 16 and 17 human embryos at a confidence level of 95% (Figure 3.1.11). Additionally, CS and postmenstrual gestational age of CS 12–15 human embryos did not correlate with each other ($r=0.251$; $P=0.101$). In contrast, the correlation between CS and postmenstrual gestational age of CS 16 and 17 human embryos was moderate and statistically

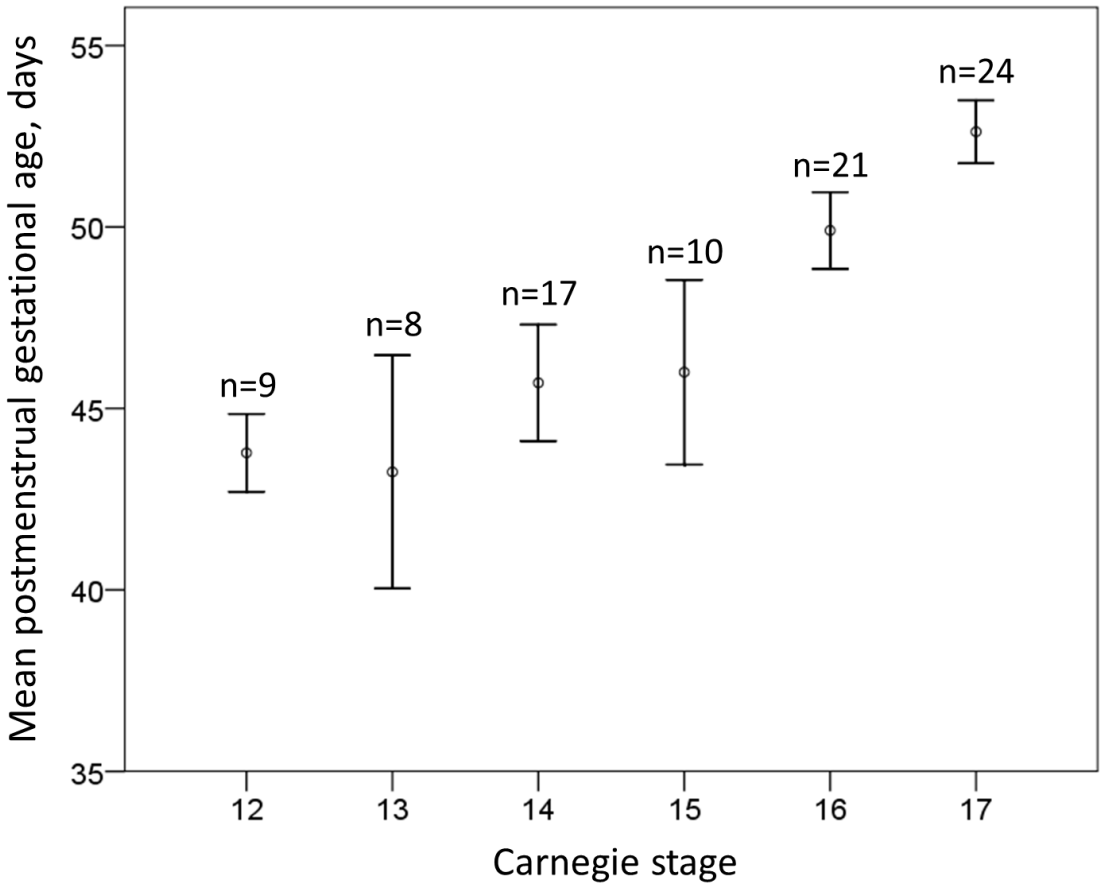


Figure 3.1.11. Relationship between postmenstrual gestational age and Carnegie stages. Human embryos obtained for the present study were split into groups depending on the Carnegie stage they belonged to. For each group, a mean postmenstrual gestational age in days (open circles) and a 95% CI (error bars) were calculated and plotted against corresponding Carnegie stages. The number of human embryos obtained for each Carnegie stage is indicated above the error bars.

highly significant (r=0.536; P<0.001). Thus, postmenstrual gestational age cannot be used to judge about the developmental stage of the human embryos under CS 16, which is crucial since the first human HSCs are detected in the human embryo starting from CS 14.

3.1.12. Discussion

Although the embryonic development of HSCs has been studied in various vertebrate species, the generation of the first HSCs during human embryogenesis remains an unexplored topic. The present concept of the embryonic development of the human haematopoietic system is primarily based on morphological and *in vitro* studies (Oberlin *et al.*, 2002; Tavian *et al.*, 1996; Tavian *et al.*, 1999; Tavian *et al.*, 2001). Here, systematic analysis of the spatio-temporal distribution of HSCs in the early human embryo was performed by employing the *in vivo* long-term repopulation assay. It has been shown that the AGM region, more specifically the dorsal aorta, is the first generator of HSCs in the human embryo. Interestingly, while in the mouse embryo definitive HSCs appear concomitantly in the AGM region, yolk sac, umbilical cord and placenta (de Bruijn *et al.*, 2000; Gekas *et al.*, 2005; Kumaravelu *et al.*, 2002; Muller *et al.*, 1994; Ottersbach and Dzierzak, 2005); in the human embryo, the timing of HSC appearance in these sites is clearly resolved, possibly due to considerably longer development of the human embryo. Additionally, in contrast to the mouse embryo (de Bruijn *et al.*, 2000), early human embryonic HSCs have never been detected in the umbilical cord.

As expected, HSC activity in the AGM region precedes that in the liver, which emerges there not before CS 17. The present data highlight the importance of *in vivo* long-term repopulation assays, since previous immunohistological and *in vitro* studies suggested that HSCs appear in the human embryonic liver around CS 13 (Tavian *et al.*, 1999). Recent transplantation experiments have shown that HSCs colonise the human embryonic liver around week 7–8 of development (Oberlin *et al.*, 2010), which corresponds to the findings of the present study.

Upon transplantation into NSG mice, early human embryonic HSCs could easily provide long-term multilineage haematopoietic engraftment. An unexpected finding was the progressive growth in the levels of repopulation with human haematopoietic cells. The percentage of human $CD45^+$ cells could reach up to 90% of total recipient mouse blood leukocytes by month 8 after transplantation. Previous reports have shown that transplantation of many human umbilical cord blood or adult bone marrow HSCs into immunodeficient mice results in blood chimerism below 50% at week 10 after transplantation and frequently decreases after several months (Cashman et al., 1997; Ishikawa et al., 2005; Liu et al., 2010). A recent study from John Dick's laboratory has shown that 10–20 umbilical cord blood HSCs transplanted per NSG recipient mouse are able to provide up to 40% repopulation in the blood by month 8 after transplantation (Notta et al., 2011). In the next result subchapter, data are provided showing that in the majority of experiments early human embryonic HSCs have been transplanted in a limiting dose (one or two HSCs per recipient mouse). This suggests that early human embryonic HSCs are much more potent in terms of long-term multilineage repopulation than their counterparts

from neonatal and adult tissues. The progressive growth in the levels of repopulation with human haematopoietic cells upon the transplantation with early human embryonic HSCs might be the result of the gradual productivity enhancement of one or two transplanted HSCs or the result of the amplification of HSC numbers. The data in section 3.1.9 suggest that early human embryonic HSCs amplify in the bone marrow of primary recipient mice. In more detail the question on the regenerative potential of early human embryonic HSCs is addressed in result subchapter 3.2.

The findings of the present study contradict a recent report that the human placenta harbors HSCs already from week 6 of development (Robin *et al.*, 2009) and demonstrate that the human placenta acquires HSC activity long after the emergence of the first HSCs in the AGM region. Of note, in the mouse embryo, definitive HSCs appear in the placenta concomitantly with the AGM region (Gekas *et al.*, 2005; Ottersbach and Dzierzak, 2005). In their study, Robin *et al.* (2009) have not provided sufficient evidence that placentas from early human embryos contained HSCs. To claim that some tissue possesses HSC activity, it is required to show that candidate HSCs within this tissue are capable of long-term multilineage haematopoietic differentiation and self-renewal. Robin *et al.* (2009) do not provide these data for the experiments with the early human placenta since in these cases the authors used PCR analysis to detect human haematopoietic engraftment in NOD/SCID recipient mice. Considering the sensitivity of the PCR analysis employed (one human cell in 100000 mouse cells) and a very early time point of donor contribution analysis (starting from week 5 after transplantation), a persistence of small numbers of some placenta-derived haematopoietic progenitor or nonhaematopoietic cells in recipient mice could

not be excluded. However, the presence of HSCs in the human placenta was convincingly shown starting from week 9 of development. In those experiments, the authors employed the long-term repopulation assay and flow cytometry but not PCR analysis to detect human multilineage haematopoietic engraftment in mice transplanted with human placental cells (Robin *et al.*, 2009).

3.2. Regenerative Potential of Early Human Embryonic HSCs

3.2.1. Introduction

From clinical studies, it is known that patient survival after HSC transplantation is improved by increasing the transplanted cell dose, which can be explained by the faster haematological and immunological recovery, lower chance of the bone marrow failure and a more robust graft-versus-leukaemia effect (Mehta *et al.*, 1996). If we assume that the human HSC pool is as heterogeneous as that in the mouse (Benz *et al.*, 2012; Dykstra *et al.*, 2007a; Sieburg *et al.*, 2006), the better survival of patients transplanted with higher cell doses may also be explained by engraftment with sufficient numbers of HSCs possessing a high regenerative potential. Although it is of an enormous clinical significance, at present nothing is known about low- and high-level repopulating human HSCs. Transplantation of single HSCs may elucidate this question. For obvious reasons, this can be done only in xenotransplantation experiments.

Analysing the results of studies which involved the transplantation of human HSCs into immunodeficient mouse recipients in a limiting dose, one could conclude that human high-level repopulating HSCs do not exist since only low-level engraftments with human haematopoietic cell levels far below 1% of total recipient blood leukocytes were observed (Majeti *et al.*, 2007; Notta *et al.*, 2011). However, it is possible that human HSCs with a high regenerative potential cannot be revealed in mouse recipients due to suboptimal self-renewal and differentiation signals as a result of the incompatibility between mouse cytokines and corresponding human HSC receptors and/or inadequate levels of compatible mouse cytokines (reviewed by

Manz, 2007). It is still not clear how to explain human high-level haematopoietic engraftments in which upon the transplantation with 10–20 umbilical cord blood HSCs the level of human $CD45^+$ cells in the recipient mouse peripheral blood may reach up to 40% of total leukocytes by month 8 after transplantation (Notta *et al.*, 2011). This may be a cumulative repopulation effect of the transplantation with many low-level repopulating HSCs.

In the preceding result subchapter, it has been shown that the high-level human haematopoietic repopulation of NSG recipient mice can easily be achieved upon the transplantation of human AGM region, yolk sac or embryonic liver HSCs. In these experiments, the percentage of human $CD45^+$ cells could reach up to 90% of total recipient mouse blood leukocytes by month 8 after transplantation, suggesting that the early human embryonic haematopoietic tissues may contain high-level repopulating HSCs. An alternative explanation for the above observations may be a cumulative repopulation effect due to the presence in the human AGM region, yolk sac or embryonic liver of many low-level repopulating HSCs. These two possibilities are explored below.

3.2.2. Experimental Approach

The main goal of this part of the study was to quantify HSCs in the early human embryo and to assess their regenerative potential. To achieve this, the long-term repopulation assay was combined with the limiting dilution analysis. When it was required to calculate numbers of daughter HSCs produced by early embryonic HSCs in the bone marrow of primary recipient mice, the secondary repopulation assay was

performed in conjunction with the limiting dilution analysis. Secondary transplantations were also performed to establish the immunophenotype of daughter HSCs and to assess their migratory abilities. Since the vast majority of the human embryos used in the present study were very early human embryos containing HSCs only in the AGM region, this result subchapter is mainly focused on human AGM region HSCs.

3.2.3. Human AGM Region Contains One or Two HSCs at a Time

Upon transplantation, a single HSC can restore haematopoiesis of a myeloablated recipient, which has been shown for both mouse and human HSCs (Notta *et al.*, 2011; Osawa *et al.*, 1996). This allows estimating the frequency of HSCs in a cell population of interest using the limiting dilution analysis (Conneally *et al.*, 1997; Szilvassy *et al.*, 1990). Given that some HSCs, for example, cycling (Fleming *et al.*, 1993) are not able to engraft a recipient, the term 'competitive repopulating unit' has been introduced to distinguish between the true frequency of HSCs and the frequency of HSC determined by transplantation (Szilvassy *et al.*, 1990). It is also not clear whether all human HSCs or just a subset called SCID-repopulating cells are able to engraft immunodeficient mice (Larochelle *et al.*, 1996). Therefore, evaluating the frequency of HSCs in the human AGM region or in any other tissue of interest, it is required to keep in mind that the true frequency of HSCs may be higher than that determined by the long-term repopulation assay and the limiting dilution analysis.

In my experiments, a single cell suspension prepared from the human AGM region was split into equal portions and transplanted into two to four sublethally irradiated

NSG recipient mice. In 16 out of 17 experiments, HSC transplantations were performed at a limiting dose as only some recipients showed human long-term multilineage haematopoietic repopulation. Only in one experiment, both recipients have been found engrafted with human HSCs (Table 3.2.1). To perform limiting dilution analysis, a software programmed to handle small number of replicates and nonlinear situations was used (Hu and Smyth, 2009). Before the limiting dilution analysis was carried out, it had been confirmed that the data fitted the single-hit Poisson model (χ_1^2=0.087, P=0.768), demonstrating consistency between the 17 experiments and justifying the pooling of the data to calculate the frequency of HSCs in the human AGM region (Hu and Smyth, 2009). The model calculations showed that CS 14–17 human AGM regions contain on average 1.7 (95% CI: 1.1–2.6) HSCs per e.e (Figure 3.2.1). Remarkably, in the mouse AGM region at comparable developmental stages the number of detectable HSCs is very close to that in the human AGM region (Kumaravelu *et al.*, 2002).

3.2.4. Early Human Embryonic HSCs Possess an Enormous Regenerative Potential

Statistically, human long-term multilineage haematopoietic repopulation in each engrafted recipient mouse transplanted with human AGM region cells must have arisen mainly from one HSC. As it has been shown in section 3.1.9., the transfer of the bone marrow from one primary recipient mouse into six to 20 secondary recipient mice resulted in sustainable human HSC engraftment, suggesting that human AGM region HSCs propagated in the bone marrow of primary recipients. However, there might be an alternative possibility that the human AGM region contained some

Experiment No.	Dose Transplanted, e.e./mouse	Engrafted Mice/ Transplanted Mice
1	0.5	1/2
2	0.5	1/2
3	0.5	1/2
4	0.5	1/2
5	0.5	1/2
6	0.5	1/2
7	0.5	1/2
8	0.5	1/2
9	0.5	2/2
10	0.33	1/3
11	0.33	1/3
12	0.33	1/3
13	0.33	1/3
14	0.33	1/3
15	0.33	1/2
16	0.33	2/3
17	0.25	2/4

Table 3.2.1. Primary data used for the limiting dilution analysis to establish the frequency of HSCs in the human AGM region. Data shown are the number of engrafted NSG mice compared to the number of transplanted NSG mice in the long-term repopulation experiments performed with the AGM regions possessing HSC activity.

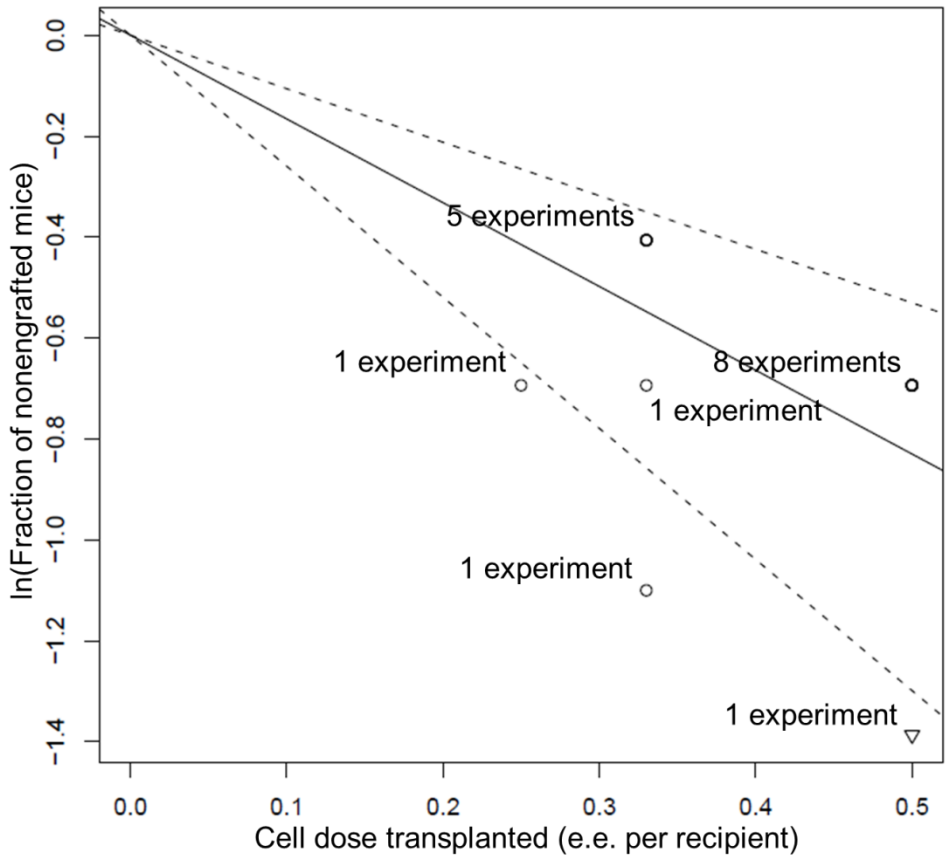


Figure 3.2.1. Results of the limiting dilution analysis establishing the frequency of HSCs in the human AGM region. In each experiment in which AGM region HSCs were detected, the natural logarithm of the fraction of nonengrafted mice was plotted against the dose of donor tissues transplanted per recipient. This is shown by the open circles. The down-pointing triangle represents the data value with zero negative response (two out of two recipients were found engrafted with human HSCs). The solid diagonal line indicates the mean value of HSC per cell dose transplanted. The dotted lines show the 95% CI. The limiting dilution model was fitted to the data in Table 3.2.1. Note that λ, a number of HSCs transplanted per recipient mouse, can be calculated from the formula λ=–ln(Fraction of nonengrafted mice). From here, –ln(0.37)≈1, which means that the dose of transplanted cells yielding ≈37% of nonengrafted recipient mice contains one HSC. To establish the dose of human AGM region cells containing a single HSC, draw an imaginary horizontal line from the *y* axis at –1 to intercept the solid diagonal line. Then, draw an imaginary vertical line from the point of interception of the imaginary horizontal line and the solid diagonal line until it cuts the *x* axis at ≈0.6, which is the dose of human AGM region cells containing one HSC.

HSCs which remained dormant in the bone marrow of primary recipient mice but could be activated upon retransplantation. To test this, bone marrow cells from two coxal bones, two femurs and two tibiae were collected from nonrepopulated primary recipient mice (Figure 3.2.2 A, mice a and b) from five independent experiments in which at least one other recipient mouse had been found engrafted with human AGM region HSCs (Figure 3.2.2 A, mouse c). In each case, the cells were transplanted into sublethally irradiated secondary NSG recipient mice. None of the secondary recipients showed human haematopoietic engraftment, confirming that the human AGM region contained only one or two HSCs and that these rare HSCs propagated in the bone marrow of primary recipients. This explains the observed progressive growth in the levels of repopulation with human haematopoietic cells. Other embryonic haematopoietic tissues have also been assessed for the presence of dormant HSCs. Secondary transplantations of the bone marrow from nonengrafted recipient mice transplanted with yolk sac, liver, umbilical cord and placental cells were also unsuccessful. For each tissue, two to five independent experiments were performed.

To evaluate the number of daughter HSCs generated by a single transplanted human AGM region HSC, primary recipient mouse bone marrow cells were subject to the limiting dilution analysis performed in conjunction with the secondary repopulation assay. The bone marrow from engrafted primary recipients was harvested from the sternum, humeri, ulnae, radii, coxal bones, femurs and tibiae, which composes approximately 2/5 of total bone marrow (Boggs, 1984), pooled and transplanted in serial dilutions into secondary recipients. In the first experiment, which was

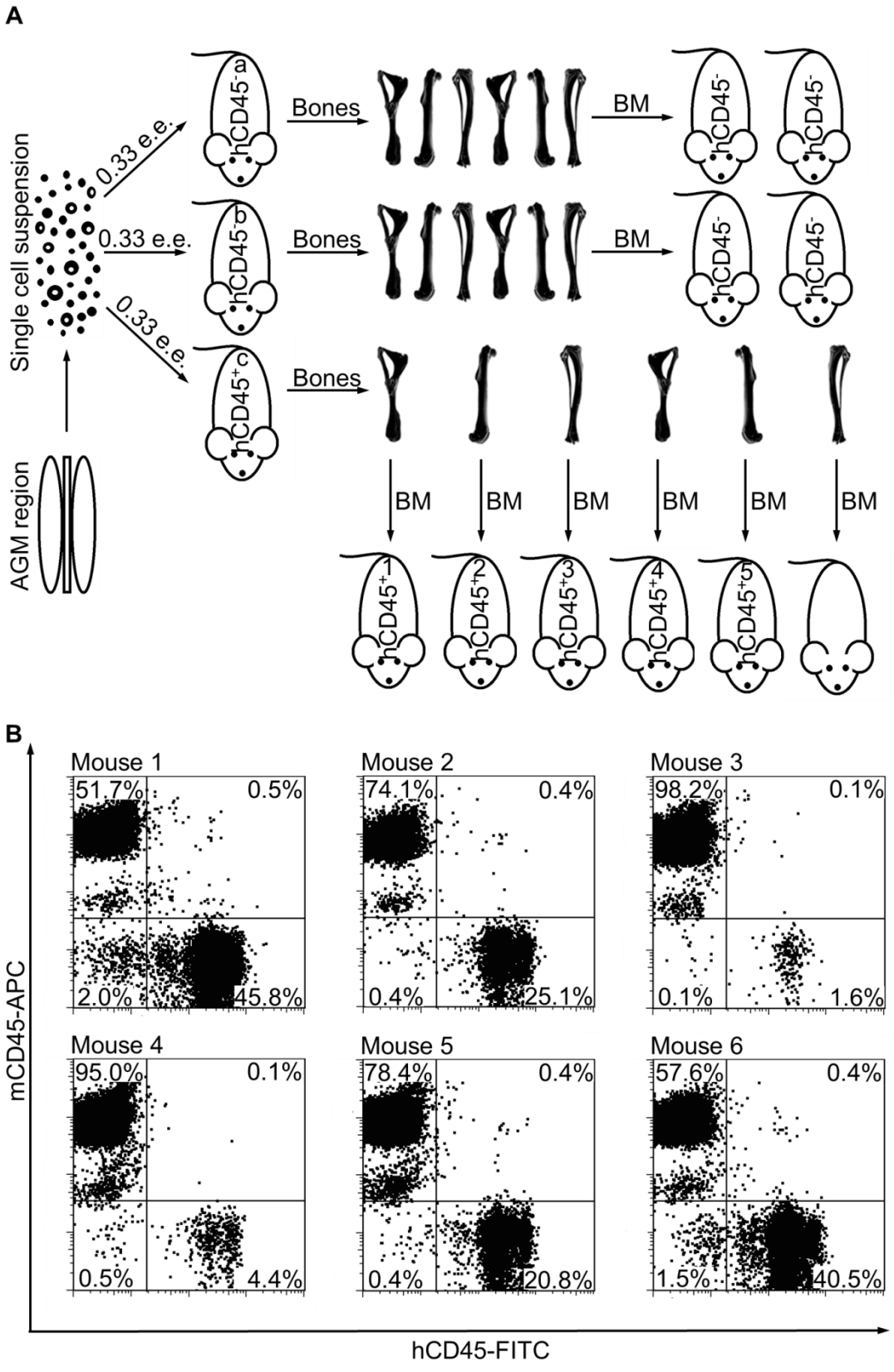

A
Single cell suspension
AGM region
0.33 e.e.
0.33 e.e.
0.33 e.e.
Bones
Bones
Bones
BM
BM
BM
BM
BM
BM
BM
B
Mouse 1
51.7%
0.5%
2.0%
45.8%
Mouse 2
74.1%
0.4%
0.4%
25.1%
Mouse 3
98.2%
0.1%
0.1%
1.6%
Mouse 4
95.0%
0.1%
0.5%
4.4%
Mouse 5
78.4%
0.4%
0.4%
20.8%
Mouse 6
57.6%
0.4%
1.5%
40.5%
mCD45-APC
hCD45-FITC

Figure 3.2.2. Experimental strategy employed to confirm the expansion of human AGM region HSCs in the bone marrow of primary recipient mice. (A) AGM region cells obtained from a CS 15 human embryo were transplanted into three sublethally irradiated NSG recipient mice (0.33 e.e. per recipient). Only one of the three recipients showed human long-term multilineage haematopoietic repopulation (mouse c). It was confirmed by secondary transplantation that the other two recipients (mice a and b) contained no activatable HSCs. To test if the single human HSC that repopulated mouse c had generated daughter HSCs which could spread across the recipient bone marrow, bone marrow cells from two coxal bones, two femurs and two tibiae were harvested and separately transplanted into six secondary recipients (mice 1–6). Secondary transplantations were performed 4 months after the primary transplantation. BM, bone marrow. **(B)** Representative flow cytometry plots show haematopoietic repopulation of human origin in the peripheral blood of the 6 secondary recipients 3 months later. Two independent experiments were performed.

performed 8 months after primary transplantation, the lowest dose transplanted per secondary recipient mouse was 1/75 of total primary recipient mouse bone marrow. All secondary recipients achieved human long-term multilineage haematopoietic repopulation. In the second experiment, the dose was decreased further to 1/300 of total primary recipient mouse bone marrow per secondary recipient mouse. The experiment was performed 7 months after primary transplantation. Again, all secondary recipients showed engraftment with human HSCs. In this experiment, a secondary transplantation of primary recipient mouse spleen cells was also performed, resulting in human long-term multilineage haematopoietic repopulation in four out of four secondary recipient mice transplanted. Thus, one HSC from the human AGM region is capable of generating at least 300 daughter HSCs upon transplantation into sublethally irradiated NSG mice (data are not shown since more definitive data are presented below for similar experiments).

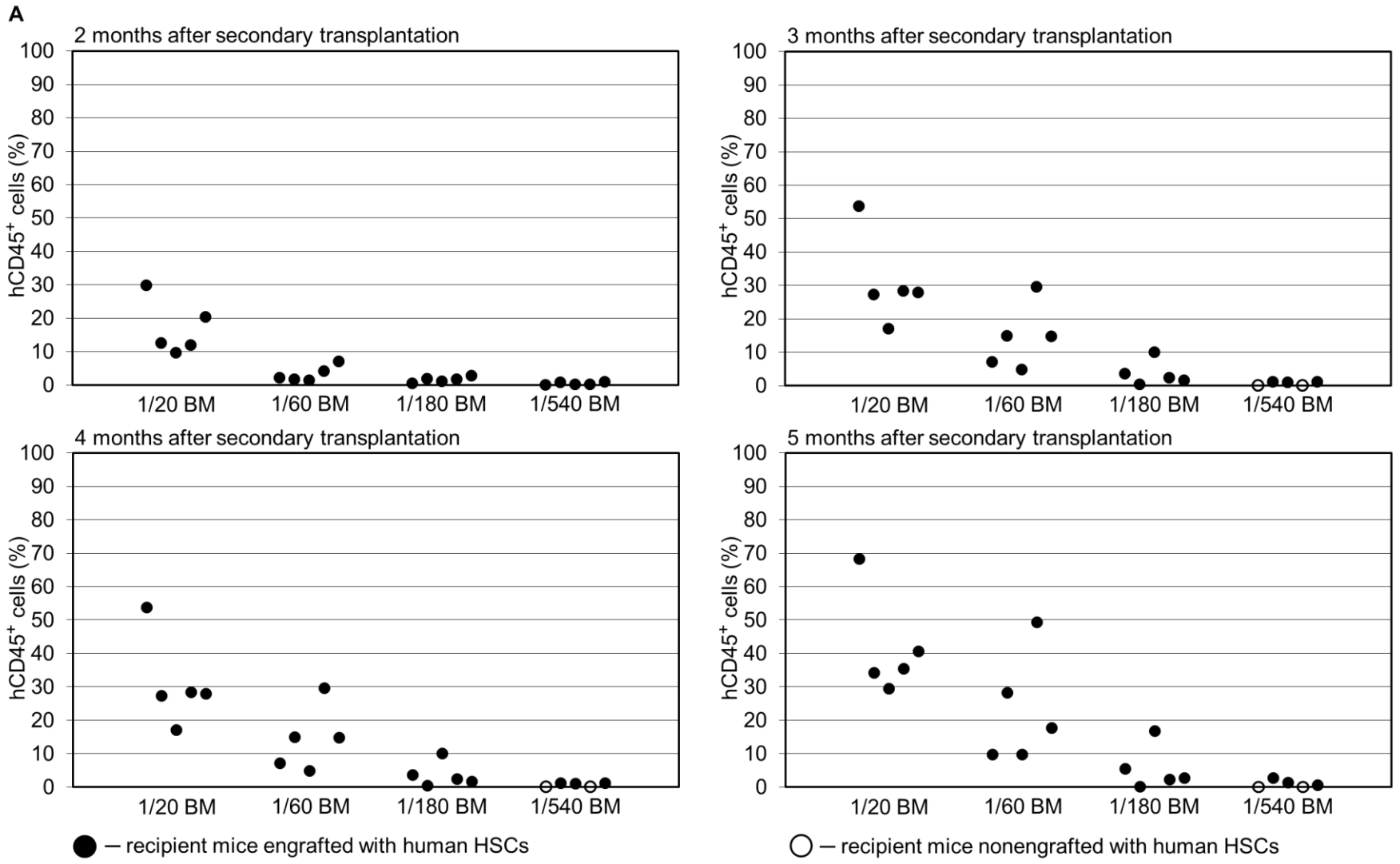
Since a limiting dose had not been found in the two above experiments, three additional independent experiments were performed at a larger scale to assess the regenerative potential of a single human AGM region HSC. Primary recipient mice used as donors in these secondary transplantations were chosen from experiments in which only one out of three primary recipients transplanted with equal numbers of human AGM region cells had been found repopulated with human haematopoietic cells. This condition was introduced to decrease the probability that the primary recipient mice had been engrafted with more than one HSC, which could skew the calculations of daughter HSC numbers. As a result, the probability was decreased to 6.3%, as computed using the Poisson distribution formula and the theory of

probability ($P_{(x>1;\lambda=0.406)}$=1–($e^{-\lambda}\lambda^0/0!$)– ($e^{-\lambda}\lambda^1/1!$), where x is a Poisson random variable, e is the base of the natural logarithm, which is 2.718, and λ is an average number of HSCs transplanted per recipient mouse, which was calculated from the formula λ=–ln(Fraction of nonengrafted mice)). In each secondary transplantation experiment, 20 sublethally irradiated NSG recipient mice were split into four experimental groups and transplanted with primary recipient mouse bone marrow cells at doses ranging from 1/20 to 1/1620 of total bone marrow per mouse. Starting from month 2 after transplantation, the recipient mice were bled by tail vein nicking every month, and the percentage of human $CD45^+$ cells was assessed. The limiting dilution analysis was performed with the values obtained at month 4 after transplantation, when the progeny of human short-term repopulating haematopoietic cells is not detectable any more (Notta *et al.*, 2011). The mice were considered as engrafted with human HSCs if at least 0.1% of total recipient blood leukocytes were of human origin, with both myeloid and lymphoid cells present. Often, others employ less stringent criteria for human HSC engraftment and consider NSG mice as engrafted if at month 4 after transplantation the bone marrow but not blood content of human $CD45^+$ cells is at least 0.1% (Notta *et al.*, 2010).

The first large-scale secondary transplantation experiment was performed 5 months after primary transplantation of AGM region cells. At that time point, the percentage of human $CD45^+$ cells in the blood and the bone marrow of primary recipient mouse was 5% and 31% cells, respectively. As assessed at month 4 after transplantation, all secondary recipients transplanted with primary recipient mouse bone marrow cells at doses 1/20, 1/60 or 1/180 of total bone marrow per mouse achieved human long-term

multilineage haematopoietic engraftment with average percentages of human leukocytes in the blood 31±14%, 14±10% and 3.6±3.8%, respectively. Two out of five secondary recipient mice transplanted with 1/540 of total primary recipient mouse bone marrow per mouse were found nonrepopulated with human haematopoietic cells. The percentage of human leukocytes in the blood of the other three secondary recipients was on average 1.1±0.058% of total blood leukocytes (Figure 3.2.3 A). Employing the limiting dilution analysis, it has been computed that during a 5-month period a single human AGM region HSC produced 604 (95% CI: 254–1440) daughter HSCs (Figure 3.2.3 B).

The second large-scale secondary transplantation was carried out 9 months after primary transplantation of AGM region cells. At that time point, the percentage of human $CD45^+$ cells in the blood and the bone marrow of the primary recipient was 50% and 96%, respectively. As assessed at month 4 after transplantation, all secondary recipient mice transplanted with bone marrow cells from the primary recipient mouse at doses 1/60 and 1/180 of total bone marrow per mouse achieved human long-term multilineage haematopoietic engraftment with average percentages of human leukocytes in the blood 6.7±7.5% and 5.0±7.9%, respectively. One out of five secondary recipient mice transplanted with 1/540 of total primary recipient mouse bone marrow per mouse was found nonrepopulated with human haematopoietic cells. The percentage of human leukocytes in the blood of the other four secondary recipients was on average 0.23±0.068% of total blood leukocytes. In group 4, secondary recipient mice were transplanted with 1/1620 of total primary recipient mouse bone marrow per mouse. Only two out of five mice survived in this


A
2 months after secondary transplantation
3 months after secondary transplantation
4 months after secondary transplantation
5 months after secondary transplantation
hCD45+ cells (%)
100
90
80
70
60
50
40
30
20
10
0
1/20 BM
1/60 BM
1/180 BM
1/540 BM
● – recipient mice engrafted with human HSCs
○ – recipient mice nonengrafted with human HSCs

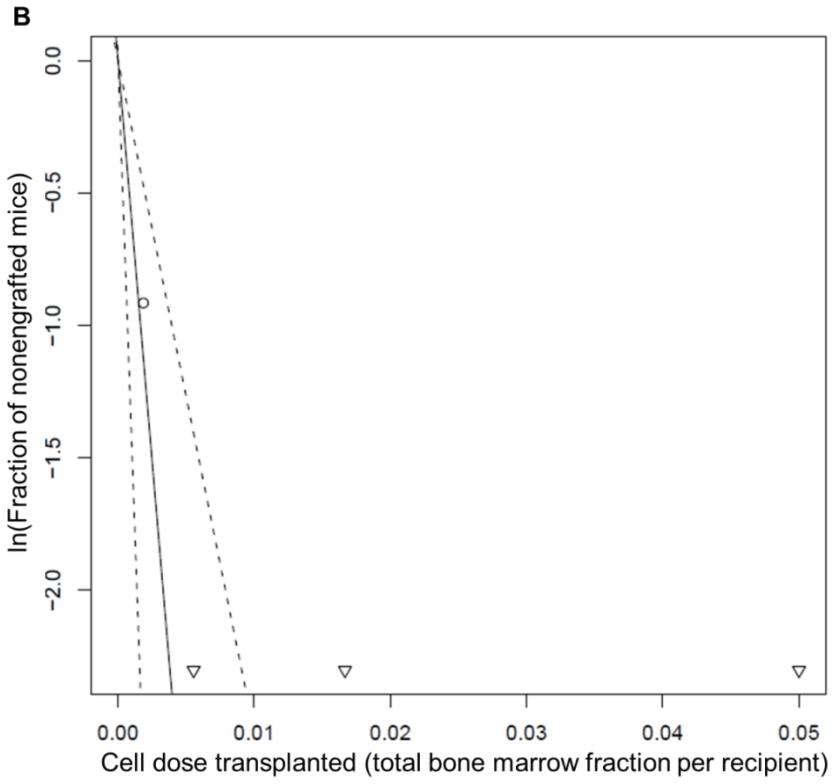


Figure 3.2.3. Results of large-scale secondary transplantation experiment 1 confirming the extensive propagation of human AGM region HSCs in the bone marrow of primary recipient mice. (A) Five months after primary transplantation, bone marrow cells from a primary recipient mouse repopulated with a single human AGM region HSC were harvested, pooled and transplanted into secondary recipients in four serial dilutions. The peripheral blood of secondary recipients was analysed for human $CD45^{+}$ cell contribution 2, 3, 4 and 5 months after transplantation. The ratios below the charts indicate the fraction of total bone marrow transplanted per recipient mouse. The circles show individual recipient mice. BM, bone marrow. **(B)** Performing the limiting dilution analysis, the natural logarithm of the fraction of nonengrafted mice in each of the four experimental groups was plotted against the dose of bone marrow cells transplanted per recipient. The down-pointing triangles represent the data values with zero negative response (five out of five recipients were found engrafted with human HSCs). The solid diagonal line indicates the mean value of HSC per cell dose transplanted. The dotted lines show the 95% CI. The limiting dilution model was fitted to the data obtained at month 4 after secondary transplantation. See Figure 3.2.1 for a detailed explanation how to read log-fraction plots.

group, and neither of them were found engrafted with human HSCs. Employing the limiting dilution analysis, it could be computed that during a 9-month period a single human AGM region HSC produced 729 (95% CI: 313–1699) daughter HSCs.

The third large-scale secondary transplantation experiment was performed 6 months after primary transplantation of AGM region cells. At that time point, the percentage of human $CD45^+$ cells in the blood and the bone marrow of primary recipient mouse was 62% and 92%, respectively. As assessed at month 4 after transplantation, all secondary recipients transplanted with primary recipient mouse bone marrow cells at doses 1/60, 1/180, 1/540 and 1/1620 of total bone marrow per mouse achieved human long-term multilineage haematopoietic repopulation with average percentages of human leukocytes in the blood 54±16%, 44±6.1%, 29±12% and 16±6.8%, respectively. Thus, during a 6-month period a single human AGM region HSC produced at least 1620 daughter HSCs (Figure 3.2.4).

3.2.5. Regenerative Potential of Human HSC Gradually Decreases During Ontogeny

To evaluate the regenerative potential of human HSCs from sources other than the AGM region, four large-scale secondary transplantation experiments were performed with the bone marrow from primary recipient mice engrafted with HSCs from the human embryonic liver (1 experiment), the midgestation placenta (1 experiment) and the umbilical cord blood (2 experiments). As in the above experiments, bone marrow cells from the primary recipients were harvested from the sternum, humeri, ulnae, radii, coxal bones, femurs and tibiae, pooled and transplanted in serial dilutions into

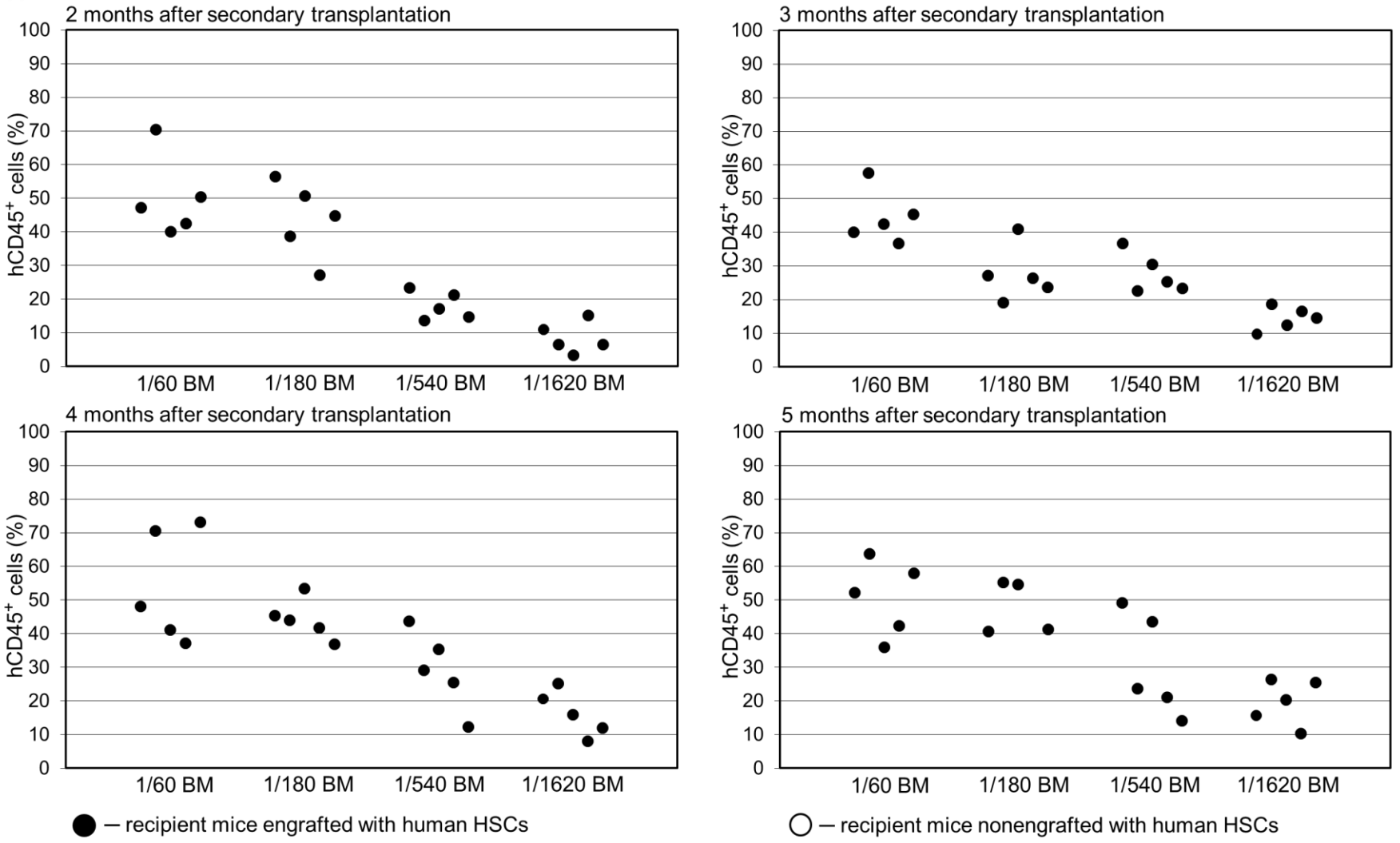

2 months after secondary transplantation
3 months after secondary transplantation
4 months after secondary transplantation
5 months after secondary transplantation
hCD45+ cells (%)
0
10
20
30
40
50
60
70
80
90
100
1/60 BM
1/180 BM
1/540 BM
1/1620 BM
● – recipient mice engrafted with human HSCs
○ – recipient mice nonengrafted with human HSCs

Figure 3.2.4. Results of large-scale secondary transplantation experiment 3 confirming the extensive propagation of human AGM region HSCs in the bone marrow of primary recipient mice. Six months after primary transplantation, bone marrow cells from a primary recipient mouse repopulated with a single human AGM region HSC were harvested, pooled and transplanted into secondary recipients in four serial dilutions. The peripheral blood of secondary recipients was analysed for human $CD45^+$ cell contribution 2, 3, 4 and 5 months after transplantation. The ratios below the charts indicate the fraction of total bone marrow transplanted per recipient mouse. The circles show individual recipient mice. BM, bone marrow.

sublethally irradiated secondary NSG recipient mice. Primary recipient mouse bone marrow cell doses ranged from 1/20 to 1/1620 of total bone marrow per secondary recipient mouse. The mice were considered as engrafted with human HSCs if at month 4 after transplantation at least 0.1% of total recipient blood leukocytes were of human origin, with both myeloid and lymphoid cells present.

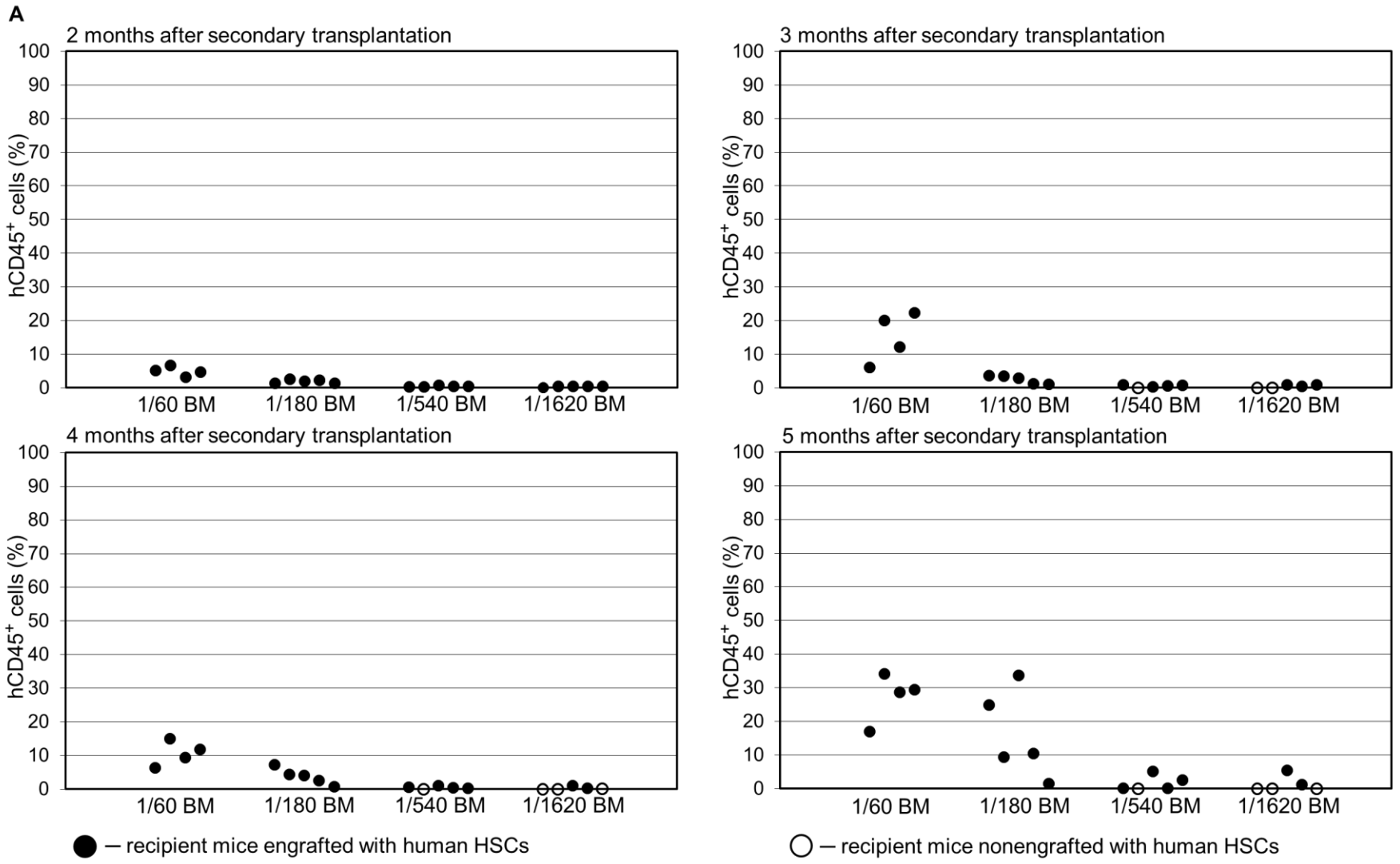
In section 3.1.3, it has been reported that in one primary transplantation experiment HSCs could be detected both in the AGM region and in the embryonic liver from the same human embryo. This was a single opportunity to compare the propagation capacity of HSCs sharing the same genetic background but derived from different human embryonic haematopoietic sites. For this, two secondary transplantation experiments combined with the limiting dilution analysis were performed in parallel using the bone marrow from primary recipient mice engrafted with human HSCs from the AGM region and the embryonic liver. It has been found that a single human AGM region HSC produced at least 1620 daughter HSCs (see the results of the third large-scale secondary transplantation experiment in the previous section, Figure 3.2.4). Unfortunately, it was not possible to estimate precisely the productivity of a single human embryonic liver HSC since the primary transplantation had not been performed in a limiting dose. Two out of two NSG recipient mice, each transplanted with 0.5 e.e. of human embryonic liver cells, achieved human long-term multilineage haematopoietic repopulation, suggesting that each of them had been engrafted with one or more human HSCs. If one assumes that in this particular case the human embryonic liver contained just two HSCs that have been equally distributed between two primary recipient mice in the transplantation course, the probability of such an

outcome would be 14%, as computed using the Poisson distribution formula and the theory of probability ($P_{(\text{Equal distribution of two HSCs between two recipients})}=(e^{-\lambda}\lambda^{1}/1!)^{2}$, where x is a Poisson random variable, e is the base of the natural logarithm, which is 2.718, and λ is an average number of HSCs transplanted per recipient mouse, which in this case is assumed 1). Thus, it is very likely that the human embryonic liver contained more than two HSCs. It is required to recognise this fact when evaluating the expansion capacity of human embryonic liver HSCs.

An assessment of human HSC numbers in the bone marrow of one of the two primary recipient mice engrafted with human embryonic liver HSCs has suggested that AGM region HSCs possess a higher regenerative potential than their counterparts in the embryonic liver. At month 6 after transplantation, the percentage of human $CD45^{+}$ cells in the blood and the bone marrow of the primary recipient mouse was 40% and 94%, respectively. As assessed at month 4 after transplantation, all secondary recipient mice transplanted with primary recipient mouse bone marrow cells at doses 1/60 and 1/180 of total bone marrow per mouse achieved human long-term multilineage haematopoietic engraftment with average percentages of human leukocytes in the blood 11±3.7% and 3.8±2.4%, respectively. One out of five secondary recipient mice transplanted with 1/540 of total primary recipient mouse bone marrow per recipient mouse was found nonrepopulated with human haematopoietic cells. The percentage of human leukocytes in the blood of the other four secondary recipients was on average 0.49±0.39% of total blood leukocytes. Three out of five secondary recipient mice transplanted with bone marrow cells from the primary recipient mouse at a dose 1/1620 of total bone marrow per mouse were

found nonengrafted with human HSCs. The percentage of human leukocytes in the blood of the other three mice was on average 0.28±0.47% of total blood leukocytes (Figure 3.2.5 A). Employing the limiting dilution analysis, it has been computed that at month 6 after primary transplantation 883 (95% CI: 401–1941) human HSCs could be detected in the primary recipient mouse bone marrow (Figure 3.2.5 B). Unfortunately, the bone marrow from the second primary recipient could not be used to confirm the above observations in a similar secondary transplantation experiment since the mouse had developed graft-versus-host disease at month 5 after primary transplantation with human embryonic liver HSCs, possibly due to occasionally observed defects in the negative selection of human T cells in the recipient mouse thymus (reviewed by Shultz *et al.*, 2007).

In one secondary transplantation experiment, the propagation capacity of human HSCs from the midgestation placenta was assessed. As it has been reported in section 3.1.10, placental cells from a 15-week-old (postmentrual gestational age) human foetus were transplanted into three sublethally irradiated NSG recipient mice at a dose of 1/30 of total placenta cells per recipient. In two such experiments, all recipient mice achieved human long-term multilineage haematopoietic engraftment of foetal origin, suggesting that each of them had been engrafted with one or more human HSCs. One of these primary recipients was used as a bone marrow donor for secondary transplantation performed 8 months after primary transplantation. At this time-point, the percentage of human $CD45^+$ cells in the blood and the bone marrow of the primary recipient was 3% and 23%, respectively. As assessed at month 4 after transplantation, only two out of five secondary recipient mice transplanted with


A
2 months after secondary transplantation
3 months after secondary transplantation
4 months after secondary transplantation
5 months after secondary transplantation
hCD45+ cells (%)
0
10
20
30
40
50
60
70
80
90
100
1/60 BM
1/180 BM
1/540 BM
1/1620 BM
● – recipient mice engrafted with human HSCs
○ – recipient mice nonengrafted with human HSCs

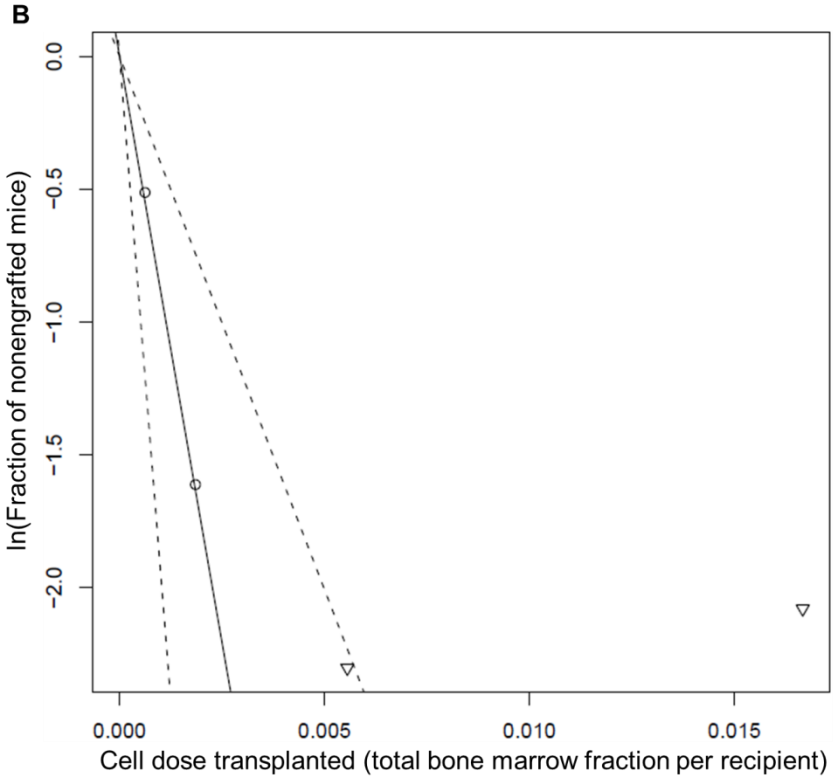


Figure 3.2.5. Results of a large-scale secondary transplantation experiment assessing the propagation capacity of human embryonic liver HSCs in the bone marrow of primary recipient mice. (A) Six months after primary transplantation, bone marrow cells from a primary recipient mouse repopulated with human embryonic liver HSCs were harvested, pooled and transplanted into secondary recipients in four serial dilutions. The peripheral blood of secondary recipients was analysed for human $CD45^+$ cell contribution 2, 3, 4 and 5 months after transplantation. The ratios below the charts indicate the fraction of total bone marrow transplanted per recipient mouse. The circles show individual recipient mice. BM, bone marrow. **(B)** Performing the limiting dilution analysis, the natural logarithm of the fraction of nonengrafted mice in each of the four experimental groups was plotted against the dose of bone marrow cells transplanted per recipient. The down-pointing triangles represent the data values with zero negative response (five out of five recipients were found engrafted with human HSCs). The solid diagonal line indicates the mean value of HSC per cell dose transplanted. The dotted lines show the 95% CI. The limiting dilution model was fitted to the data obtained at month 4 after secondary transplantation. See Figure 3.2.1 for a detailed explanation how to read log-fraction plots.

primary recipient mouse bone marrow cells at a dose 1/20 of total bone marrow per mouse achieved human long-term multilineage haematopoietic engraftment with percentages of human leukocytes in the blood 0.42% and 0.37%. All secondary recipient mice transplanted with lower bone marrow cell doses were found nonengrafted with human HSCs. Employing the limiting dilution analysis, it has been computed that at month 8 after primary transplantation 6.7 (95% CI: 1.7–26) human HSCs could be detected in the bone marrow of the recipient mouse engrafted with HSCs from the human midgestation placenta.

Finally, the regenerative potential of human umbilical cord blood HSCs was evaluated. In two independent experiments, sublethally irradiated NSG recipient mice were transplanted with 2500 of human umbilical cord blood $CD34^+$ cells each. As reported by others, this cell dose contains one or two HSCs (McDermott *et al.*, 2010). In one experiment, all five recipient mice have been found engrafted with human HSCs. In the other experiment, three out of five recipients achieved human long-term multilineage haematopoietic repopulation. The average percentage of human leukocytes in the blood of the recipients engrafted with human umbilical cord blood HSCs was 0.59±0.45%, as assessed at month 4 after primary transplantation. Two of these recipients (one from each experiment) were used as bone marrow donors for secondary transplantation experiments performed 4 months after primary transplantation with a goal to estimate human HSC numbers in the bone marrow of the primary recipients. The percentage of human $CD45^+$ cells in the blood and the bone marrow of these two mice was 0.19% and 5.1% (mouse 1) and 0.54% and 6.2% (mouse 2), respectively. In both secondary transplantation experiments, human

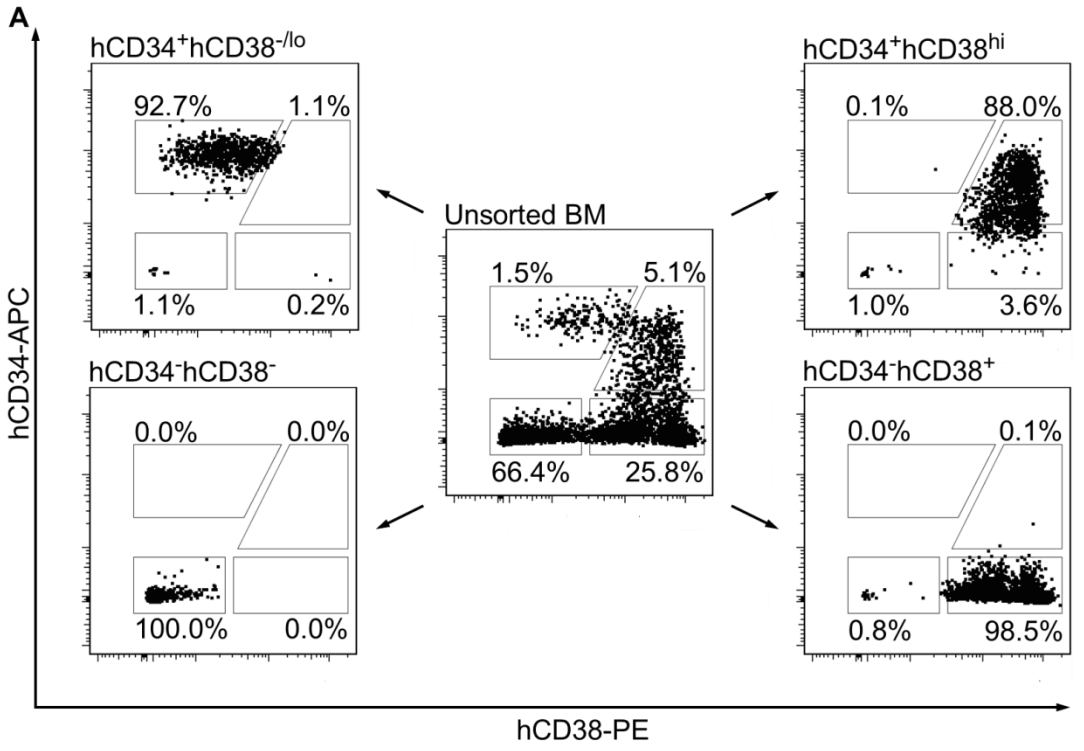
haematopoietic cells were absent in all secondary recipient mice, suggesting that in the xenotransplantation settings human umbilical cord blood HSCs transplanted in a limiting dose possess a poor regenerative potential if compared to their embryonic counterparts. Limited self-renewal capacity of HSCs from the human umbilical cord blood have been previously reported by others (Guenechea *et al.*, 2001; Liu *et al.*, 2010; McKenzie *et al.*, 2006).

3.2.6. Extensive Dissemination of Daughter HSCs in Primary Recipient Mice

The engraftment of one or two HSCs from the human AGM region results in considerable amplification of the HSC pool. It was tested if daughter HSCs stayed at one site where the lodging of the parental HSC had occurred or if they migrated and disseminated throughout the entire recipient bone marrow. Two primary NSG recipient mice repopulated with different AGM regions were euthanised. From each recipient, the bone marrow from two coxal bones, two femurs and two tibiae was harvested separately. Bone marrow cell suspensions from each bone were transplanted individually into sublethally irradiated secondary NSG recipient mice (Figure 3.2.2 A). In experiment 1, all transplanted mice showed human long-term multi-lineage haematopoietic engraftment (Figure 3.2.2 B). Consistent with this, in experiment 2, all secondary recipient mice except one were repopulated with human haematopoietic cells. Thus, one or two early human embryonic HSCs colonise the entire recipient mouse bone marrow not only through the substantial production of daughter HSCs but also through the mobilisation, migration and homing of daughter HSCs, which behaved as expected for human adult-type HSCs.

3.2.7. Daughter HSCs Are of the Canonical Phenotype

Most human adult-type HSCs reside within the $CD34^{+}CD38^{-/lo}$ cell population (Bhatia *et al.*, 1997; McKenzie *et al.*, 2006). Due to a number of reasons, it is not feasible to determine the immunophenotype of HSCs in the human AGM region using conventional high-pressure FACS. Firstly, the frequency of HSCs in the human AGM region is low (one or two HSCs per e.e.). Secondly, conventional FACS is always associated with cell losses and embryonic HSC functional damage as has been shown for the mouse AGM region (Medvisnky *et al.*, unpublished data). Thirdly, it is almost impossible to obtain at the same time and pool for a cell sorting AGM regions from several fresh human embryos at the appropriate developmental stages (CS 14–17) to overcome the above two issues. However, due to substantial self-renewal of early human embryonic HSCs in the bone marrow of engrafted primary recipient mice, it was possible to determine the immunophenotype of daughter HSCs. The bone marrow was harvested from the sternum, humeri, ulnae, radii, coxal bones, femurs, and tibiae of primary recipients engrafted with a single HSC from the human AGM region. Then, the bone marrow was immunolabelled and sorted into four cell populations based on the expression of human CD34 and CD38 antigen (Figure 3.2.6 A). Finally, each cell population was transplanted into four to five sublethally irradiated secondary NSG recipient mice. In two independent experiments, human HSCs were found exclusively within $CD34^{+}CD38^{-/lo}$ cell population (Figure 3.2.6 B). The fact that HSCs from the human AGM region give rise to daughter HSCs with an immunophenotype similar to that of human adult-type HSCs is a strong evidence that in the human embryo the AGM region is the primary source of definitive HSCs.


A
hCD34+hCD38-/lo
92.7%
1.1%
1.1%
0.2%
hCD34+hCD38hi
0.1%
88.0%
1.0%
3.6%
Unsorted BM
1.5%
5.1%
66.4%
25.8%
hCD34-hCD38-
0.0%
0.0%
100.0%
0.0%
hCD34-hCD38+
0.0%
0.1%
0.8%
98.5%
hCD34-APC
hCD38-PE

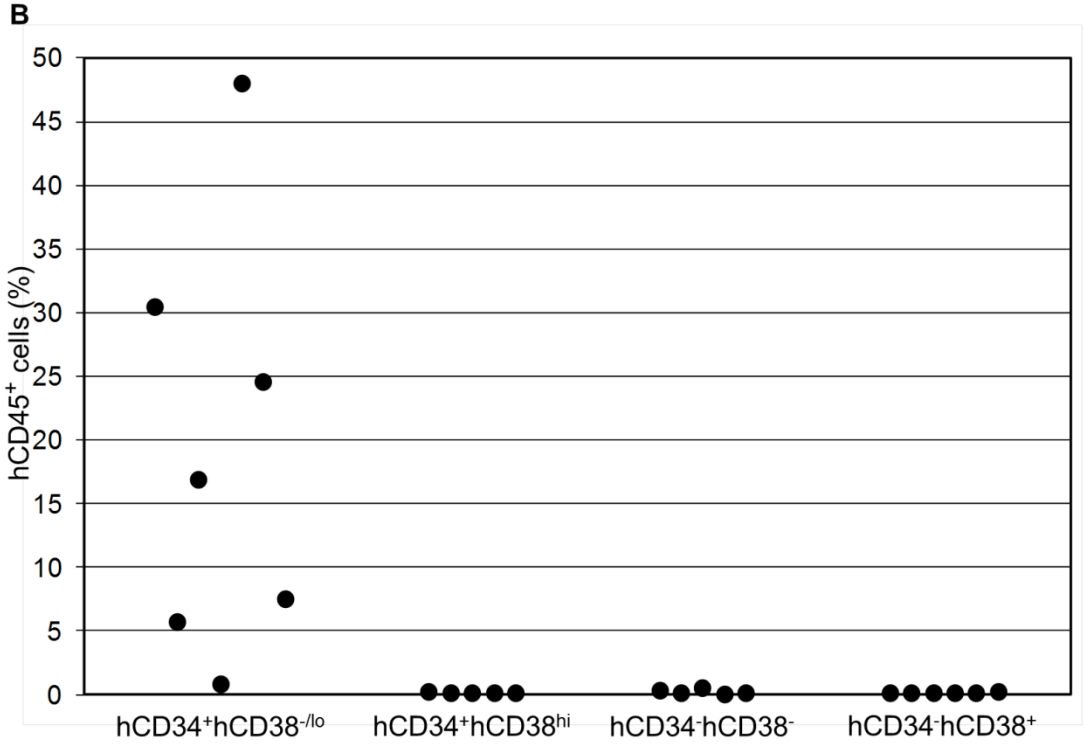

B
hCD45+ cells (%)
50
45
40
35
30
25
20
15
10
5
0
hCD34+hCD38-/lo
hCD34+hCD38hi
hCD34-hCD38-
hCD34-hCD38+

Figure 3.2.6. Immunophenotype of daughter human AGM region-derived HSCs in the bone marrow of primary recipient mice. (A) Seven months after primary transplantation, the bone marrow of NSG recipient mice repopulated with CS 16 and 17 AGM regions was sorted into four cell populations based on expression of human CD34 and CD38 antigens. Two independent experiments were performed. **(B)** Cells of each sorted population were injected into sublethally irradiated secondary recipient mice. The peripheral blood of secondary recipients was analysed for human $CD45^+$ cell contribution 2 months after transplantation. The circles show individual recipient mice. Two independent experiments were performed. BM, bone marrow.

3.2.8. Discussion

The data presented in this result subchapter show that human AGM region HSCs possess a considerably higher regenerative potential than human HSCs at later ontogenetic stages. Despite some variability in experiments, which was most likely due to genetic differences between individual human embryos, it has been demonstrated that upon transplantation into sublethally irradiated NSG mice a single HSC from the human AGM region usually produces around 600 daughter HSCs during a period of 5–9 months. This unprecedented self-renewal capacity has not been previously described for human or for mouse HSCs. It is likely as a result of this the percentage of human leukocytes in the recipient mouse blood progressively increased, reaching up to 90% of total blood leukocytes (see section 3.1.5). To achieve the same effect with human umbilical cord blood or adult bone marrow HSCs, considerably higher numbers of HSCs need to be transplanted. More recent data suggest that ≈40% content of human $CD45^+$ cells in the recipient mouse blood can be achieved 8 months after transplantation of 10–20 human umbilical cord blood HSCs per NSG mouse (Notta *et al.*, 2011).

Based on the observations from the previous experiments with human umbilical cord blood and adult bone marrow HSCs (Cashman *et al.*, 1997; Guenechea *et al.*, 2001; Ishikawa, 2005; Liu *et al.*, 2010; McKenzie *et al.*, 2006), it has been suggested that the incompatibility between mouse cytokines and corresponding human HSC receptors and inadequate levels of compatible mouse cytokines are the two main reasons for a poor expansion of human HSCs in the recipient mouse organism (reviewed by Manz, 2007). Strikingly, upon transplantation into immunodeficient

mice, human AGM region HSCs have been shown to perform considerably better than their counterparts from neonatal and adult cell sources, showing extensive self-renewal and amplification of HSC numbers. Thus, it is very likely that due to a very high propagation capacity early human embryonic HSCs can surmount the effects of the suboptimal recipient mouse bone marrow microenvironment. These may also suggest that the expansion potential of human AGM region HSCs in physiological conditions is likely to be higher than assessed by the xenotransplantation assay. However, one may propose another explanation for the robust propagation capacity of early human embryonic HSCs. Due to suboptimal microenvironment in the recipient mouse organism, it is possible that the true regenerative potential of human umbilical cord blood and adult bone marrow HSCs simply cannot be revealed by xenotransplantation and may be considerably higher than it has previously been reported (Cashman *et al.*, 1997; Guenechea *et al.*, 2001; Liu *et al.*, 2010; McKenzie *et al.*, 2006). Indeed, umbilical cord blood and adult bone marrow HSCs can successfully be used for transplantation in clinical settings (Gluckman *et al.*, 1989; Good *et al.*, 1969). As for early human embryonic HSCs, their requirements for supportive cytokines may differ from those of ontogenetically older HSCs; hence, NSG mice may be more optimal recipients for them than for human HSCs from the umbilical cord blood and adult bone marrow. Although the xenotransplantation assay may be a misleading approach for the comparison of the expansion potential of human HSCs from different sources, there are no better alternatives available at the moment.

The assessment of the regenerative potential of human HSCs from different sources has suggested that during embryo- and foetogenesis the propagation capacity of human HSCs declines. It has been found that upon transplantation into sublethally irradiated NSG mice HSCs from the human embryonic liver, midgestation placenta and umbilical cord blood produce smaller number of daughter HSCs than HSCs from the human AGM region. This is consistent with AGM region HSCs being founders of the adult haematopoietic system in the human. Since the few first HSCs emerging in the human AGM region around day 35 of development are ancestors for thousands of HSCs residing in the adult human bone marrow, it may well be imagined that during this massive expansion the daughter HSCs through some unknown mechanism lose the huge regenerative potential of the parental HSCs. Similar conclusions can be drawn from the secondary transplantation experiments. While in primary recipient mice transplanted with a single human AGM region HSC there was observed a progressive growth in the levels of repopulation with human haematopoietic cells, most likely related to the propagation of this single HSC, this was not observed to the same extent in secondary recipients transplanted with daughter HSCs. It is possible that this reflects the loss of the regenerative potential observed during human embryo- and foetogenesis. However, this may not be a replication of a biological phenomenon but just an experimental artifact due to prolonged exposure of human HSCs to suboptimal microenvironment in the recipient mouse bone marrow.

Using stochastic analyses, it has been shown that adult human HSCs replicate every 40 weeks (Catlin *et al.*, 2011). If one assumes that human AGM region HSCs do not

lose their propagation capacity upon transplantation into the mouse and undergo cell divisions regularly, it can be speculated that the extensive expansion of early human embryonic HSCs occurs through replication every 2–3.5 weeks or even more frequently, considering that HSCs also produce mature blood cells. However, this view on the behaviour of human HSCs in the recipient mouse organism is rather simplified, and it is possible that human AGM region HSCs in the mouse undergo cell divisions irregularly, for example, the main expansion steps occur shortly after transplantation, and then HSCs self-renew less frequently. In this case, the length of the cell cycle shortly after transplantation would be even shorter than discussed above, which is consistent with AGM region HSCs being founders of the adult haematopoietic system in the human.

Mathematical simulations performed by Catlin *et al.* (2011) have suggested that to prevent bone marrow failure after myeloablation before clinical HSC transplantation a minimum of 100 HSCs should be transplanted. For comparison, a single human AGM region HSC produces about 600 daughter HSCs. To alleviate the problem of the shortage of HSC donors in the absence of HSC *ex vivo* expansion strategies, it is theoretically possible to use the bone marrow from germ-free NSG mice engrafted with human AGM region HSCs as a source of high numbers of clinically transplantable human HSCs and haematopoietic progenitors. Moreover, these cells have been shown to be easily engraftable due to their outstanding capability to disseminate through the entire recipient bone marrow.

3.3. Localisation and Immunophenotypic Characterisation of Human AGM Region HSCs

3.3.1. Introduction

The observations reported in the above two result subchapters suggest that human AGM region HSCs possess an unprecedented propagation capacity considerably superseding that of human HSCs from other cell sources. Therefore, this is not only of academic interest but also of clinical importance to understand the mechanism underlying the high regenerative potential of early human embryonic HSCs. One of the ways to clarify this is by studying differential gene expression in human AGM region HSCs versus human umbilical cord blood HSCs. Although the latter can be enriched to a comparatively high purity (Notta *et al.*, 2011), at the moment there are no methods to purify HSCs from the human AGM region.

A number of reasons preclude the use of conventional high-pressure FACS to study the immunophenotype of human AGM region HSCs and to purify them. Firstly, the frequency of HSCs in the human AGM region is very low (one or two HSCs per e.e.). Secondly, conventional FACS is always associated with cell losses and embryonic HSC functional damage, as it has been shown for the mouse AGM region (Rybtsov *et al.*, unpublished data). Thirdly, it is practically impossible to overcome the above two obstacles by obtaining at the same time and pooling for a cell sorting AGM regions from several fresh human embryos at the appropriate developmental stages (CS 14–17). Hence, it is required to look at alternatives for conventional FACS.

The on-chip microfluidic FACS may be an appropriate substitute for conventional FACS. By employing this technique, it is possible to exclude cell losses using a closed-loop sorting strategy. Additionally, cell functional damage is prevented by low-pressure cell sorting. The main disadvantage of the on-chip microfluidic cell sorting is a comparatively low cell sorting speed (100 events per second), which limits the input cell number (Wang *et al.*, 2005). The precise anatomical localisation of HSCs within the human AGM region would overcome this limitation since the tissues free of HSC activity could be removed before cell sorting, decreasing the input cell number.

In section 3.1.4, it has been reported that the dorsal aorta but not the UGRs is the source of HSC activity within the human AGM region. In the mouse embryo, the vast majority of definitive HSCs were found in the AoV (Taoudi and Medvinsky, 2007). Therefore, it was reasonable to ask if this could also be extended to the human embryo. Beside the observations of the distribution of intra-aortic haematopoietic cell clusters (Minot, 1912; Tavian *et al.*, 1996), no data to support the existence of the dorso-ventral polarity in haematopoietic activity within the human dorsal aorta has been presented so far. If the dorso-ventral polarity in HSC activity within the human dorsal aorta does exist, this would allow us to progress with the on-chip microfluidic cell sorting experiments in order to find the immunophenotype of human AGM region HSCs. More notably, this would also permit us to address a number of important biological questions, the very first of which could be related to gene expression in the early human embryonic HSC niche (differential gene expression in the AoV versus the AoD).

3.3.2. Experimental Approach

To establish the existence of the dorso-ventral polarity in haematopoietic activity within the human embryonic dorsal aorta, the dorsal aorta and the surrounding mesenchyme were subdissected along the midline into the AoV and the AoD, as previously described for the mouse embryo (Taoudi and Medvinsky, 2007). The main goal of this part of the study was to assess the dorso-ventral polarity in HSCs activity. For this, single cell suspensions prepared from the ventral and the dorsal parts of the human embryonic dorsal aorta were individually transplanted into sublethally irradiated NSG recipient mice. To increase the success rate of these experiments, CS 15–17 human embryos were used. Flow cytometry and CFU-C analyses were also performed to obtain additional evidence to support the existence of the dorso-ventral polarity in haematopoietic activity within the human embryonic dorsal aorta.

3.3.3. Spatial Distribution of Haematopoietic Cells within the Human AGM Region

Five independent experiments were performed with CS 15–17 human embryos to establish the spatial distribution of haematopoietic cells within the human AGM region. The expression of CD45, a marker of all haematopoietic cells except erythrocytes and megakaryocytes, and CD34, a marker of human HSCs and haematopoietic progenitors, was assessed by flow cytometry analysis performed with the cells from the AoV, the AoD and the UGRs. The AoV and the AoD included the ventral aortic endothelium and the dorsal aortic endothelium, respectively, and the

surrounding mesenchyme. The UGRs included the gonads, the mesonephros and a portion of the mesenchyme adjacent to the aorta laterally.

The average absolute number of $CD45^+$ cells in the AoV, the AoD and the UGRs was 7280±5490, 3370±1900 and 7490±6460 cells per e.e., respectively (Figure 3.3.1 A). Although the AoV and the UGRs contained on average twice as many $CD45^+$ cells as the AoD, the difference between the data sets was not statistically significant when assessed with the parametric Paired-Samples T Test. The probability values P for the pairs the AoV versus the AoD, the AoV versus the UGRs and the AoD versus the UGRs were 0.086, 0.897 and 0.159, respectively. The use of the parametric statistical test was formally dictated by the normal distribution of the data sets, as determined with the Kolmogorov-Smirnov Test. Knowing that with small data sets the Kolmogorov-Smirnov Test may have little power to discriminate between normally and not normally distributed data (Motulsky, 2010), the nonparametric Wilcoxon Signed Ranks Test was additionally performed to compare the data sets. A statistically significant difference in the absolute number of $CD45^+$ cells was only found between the AoV and the AoD (P=0.043).

Performing flow cytometry analysis of subdissected human AGM regions, it became evident that the AoV contains a population of $CD34^{hi}CD45^{lo}$ cells (Figure 3.3.2). In the AoD and the UGRs, these cells were underrepresented or, in some embryos, even absent. The average absolute number of $CD34^{hi}CD45^{lo}$ cells in the AoV, the AoD and the UGRs was 1480±860, 150±210 and 420±460 cells per e.e., respectively (Figure 3.3.1 B). The Paired-Samples T Test revealed that the AoV contains

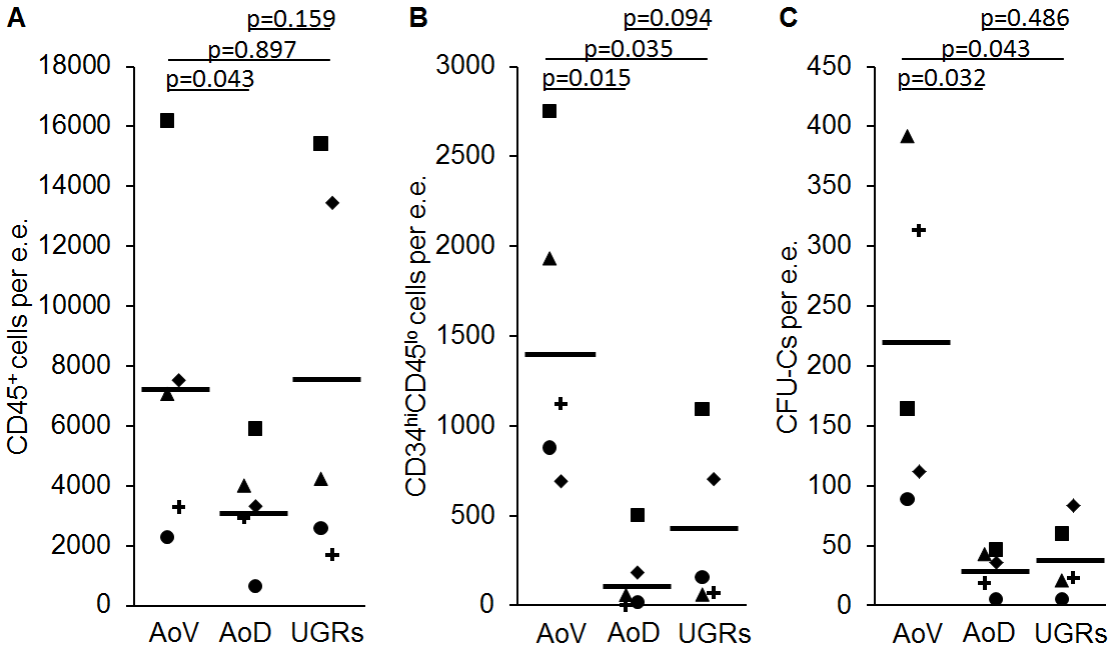


Figure 3.3.1. Absolute numbers of CD45^{+} and CD34^{hi}CD45^{lo} cells and CFU-Cs in the AoV, the AoD and the UGRs dissected from early human embryos. AGM regions from five CS 14–17 human embryos were subdissected into the AoV, the AoD and the UGRs, and individual single cell suspensions were prepared from these tissues. The number of CD45^{+} **(A)** and CD34^{hi}CD45^{lo} cells **(B)** per e.e. was assessed by flow cytometry using counting beads as an internal control. The number of CFU-Cs per e.e. was evaluated plating AoV, AoD and UGR cells into methylcellulose medium supplemented with human cytokines **(C)**. Each symbol (●, ■, ▲, + and ♦) represents the same individual human embryo. The horizontal lines indicate mean absolute values.

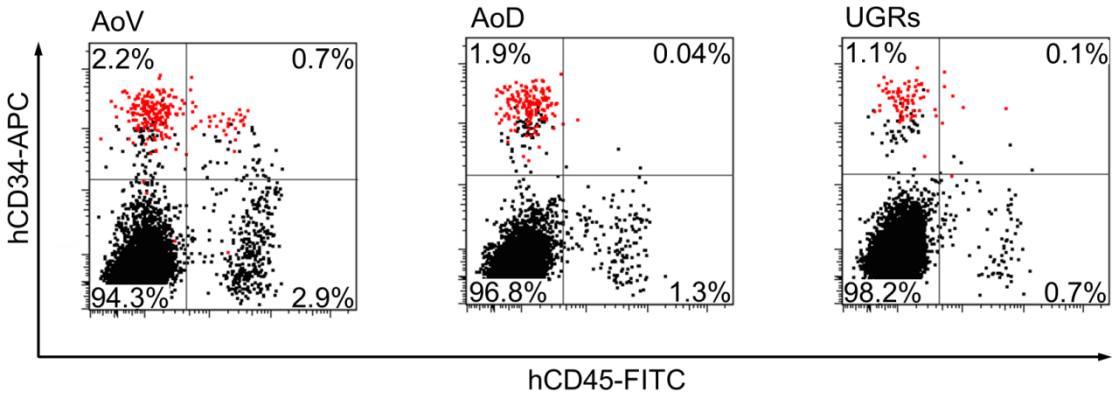


Figure 3.3.2. Expression of CD34, CD144 and CD45 by human AoV, AoD and UGR cells. The human AGM region was subdissected into the AoV, the AoD and the UGRs, and individual single cell suspensions were prepared from these tissues. After immunolabelling with anti-human CD34, CD144 and CD45 monoclonal antibodies, cells were analysed by flow cytometry. Representative flow cytometry plots show $CD34^{hi}CD144^{+}CD45^{lo}$ cell population identifiable only in the AoV but not in the AoD and the UGRs. $CD144^{+}$ cells are overlaid and shown in red.

statistically significantly more $CD34^{hi}CD45^{lo}$ cells that the AoD and the UGRs. The P values for the pairs the AoV versus the AoD and the AoV versus the UGRs were 0.015 and 0.035, respectively. There was no statistically significant difference in the number of $CD34^{hi}CD45^{lo}$ cells between the AoD and the UGRs (P=0.094).

3.3.4. Spatial Distribution of CFU-Cs within the Human AGM Region

Since the cells with HSC and haematopoietic progenitor immunophenotype had been found predominantly in the AoV, it was of interest to assess functionally the spatial distribution of haematopoietic progenitors within the human AGM region. To this end, five independent experiments were performed with subdissected AGM regions from CS 14–17 human embryos. Single cell suspensions prepared from the AoV, the AoD and the UGRs were plated into the methylcellulose medium supplemented with human cytokines. After 14 days of culture, human erythroid, myeloid and mixed haematopoietic colonies were enumerated. The average number of CFU-Cs in the AoV, the AoD and the UGRs was 210±130, 30±20 and 40±30 CFU-Cs per e.e., respectively (Figure 3.3.1 C). The Paired-Sample T Test showed a statistically significant difference in the number of CFU-Cs only between the AoV and the AoD (P=0.032). The probability values P for the pairs the AoV versus the UGRs and the AoD versus the UGRs were 0.056 and 0.486, respectively. Since the P value for the pair the AoV versus the UGRs was just slightly above 0.05 and due to the possibility that the Gaussian assumption might be violated, the Wilcoxon Signed Ranks Test was additionally performed to compare the data sets. A statistically significant difference in the absolute number of CFU-Cs between the AoV and the UGRs was confirmed with the nonparametric statistical test (P=0.043).

3.3.5. Spatial Distribution of HSCs within the Human AGM Region

In section 3.1.4, it has been reported that the dorsal aorta but not the UGRs possess HSC activity within the human AGM region. Considering the above data on the dorso-ventral polarity in the distribution of haematopoietic cells, cells with HCS immunophenotype and CFU-Cs within the human dorsal aorta, the last step was to assess the dorso-ventral polarity in HSC activity. This was tested in 10 independent transplantation experiments performed with CS 15–17 human embryos. In each case, the human dorsal aorta was subdissected into the AoV and the AoD, as previously described for the mouse AGM region (Taoudi and Medvinsky, 2007). Single cell suspensions prepared from these tissues were individually transplanted into sublethally irradiated NSG recipient mice. In 10 experiments performed, HSCs were detected on six occasions in the AoV and never in the AoD (data not shown).

3.3.6. On-Chip Microfluidic Cell Sorting of Human AGM Region Cells

Characterisation of the immunophenotype of the earliest human HSCs is crucial to elucidate the molecular mechanisms underlying the high regenerative potential of these cells. As discussed in the introduction to this result subchapter, conventional high-pressure FACS is not a method of choice to study the immunophenotype of human AGM region HSCs. The on-chip microfluidic FACS can be employed instead. However, the limitations of this method are a comparatively low cell sorting speed and, as a consequence, a limited number of input cells. After it had been established that the human AGM region HSCs reside in the ventral wall of the dorsal aorta, it became feasible to study the immunophenotype of the earliest human HSCs overcoming the issue with the limited number of input cells by removing the AoD

and the UGRs before cell sorting. The AoV from CS 14–17 human embryos contains on average 162480±125970 cells per e.e. Considering that the maximum speed of the on-chip microfluidic cell sorting is 100 events per second, it can be estimated that the isolation of a population of interest from the AoV requires about 30 min to complete. This is a reasonable duration for a cell sort and is unlikely to severely compromise cell viability and functionality.

To perform the on-chip microfluidic FACS, a Celula mvs360b instrument was employed. The machine was equipped with a 488 nm laser, a forward scatter detector and four fluorescence detectors. Due to difficulties in the compensation of the spectral overlap between different fluorochromes, only three fluorescence detectors could be used in practice (525/40, 575/25 and 624/40). The instrument could only perform a two-way sorting, which was determined by the design of the Celula mvs360b sorting chip (Figure 3.3.3).

In view of the immunophenotype of mouse AGM region HSCs (Taoudi *et al.*, 2005) and the fact that the human AoV, which exclusively harbours HSC activity within the human AGM region, contains the vast majority of human AGM region $CD144^{+}CD45^{+}$ cells, I isolated $CD144^{+}CD45^{+}$ cells from the human AoV and tested them in the long-term repopulation assay. To this end, human AoV cells were immunolabelled with anti-human CD45-FITC and CD144-PE monoclonal antibodies, resuspended in 7-AAD solution and sorted on-chip into two cell populations, $CD144^{+}CD45^{+}$ cells and all the other remaining cells. The cell populations were individually transplanted into sublethally irradiated

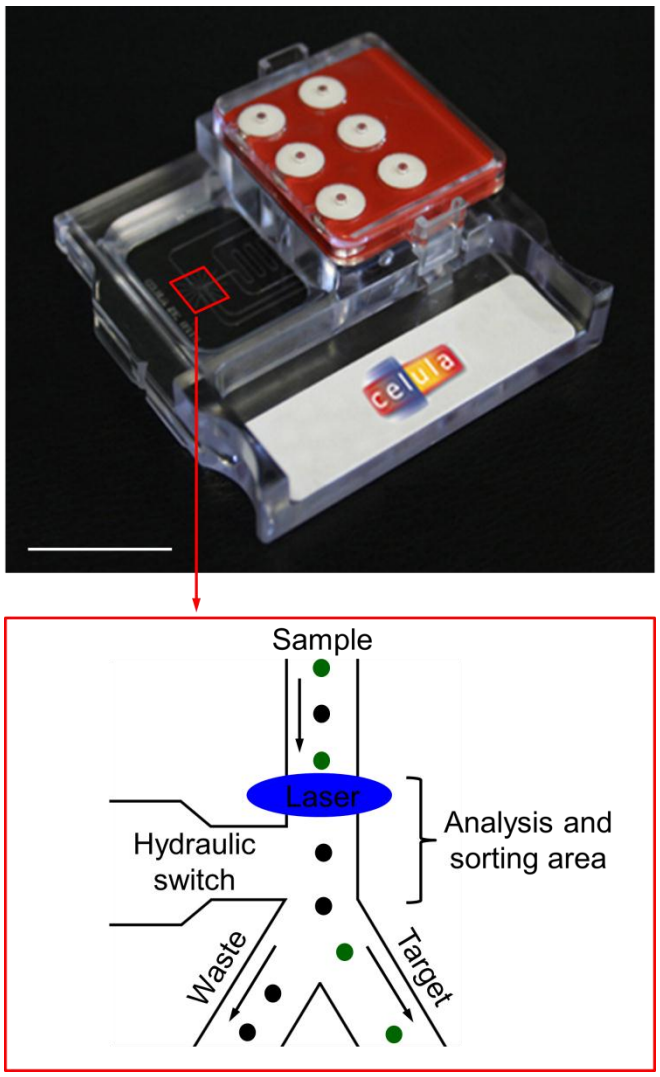


Figure 3.3.3. Design of the disposable Celula mvs360b chip for microfluidic FACS. Cells are loaded into a sample cup. Driven by a low hydraulic pressure gradient, the cells enter the system of microchannels to become focused in the centre of the sheath fluid stream and separated from each other. Entering the analysis and sorting area, the cells are exposed to the laser. In the case a target cell is detected, hydraulic pressure is applied to deviate the cell towards a target cell chamber. Irrelevant cells flow into a waste cell chamber by default. Scale bar, 10 mm.

NSG recipient mice. In total, five independent transplantation experiments were performed with sorted AoV cells from CS 15–17 human embryos. Strikingly, in all five experiments HSCs could be detected in none of the two sorted populations. Statistically, every second CS 15–17 human embryo should contain HSCs in the AGM region (see Table 3.1.1 and sections 3.1.4 and 3.3.5). The failure to detect HSC in the long-term repopulation assay might be explained by the functional impairment of human AGM region HSCs following the on-chip microfluidic cell sorting. Among the possible factors that could contribute to the loss of HSC activity might be HSC damage by the laser or due to toxic effects of some substances released by the chip. To test this, three technical experiments were set up with AGM region cells from day 11.5 Ly5.2/Ly5.2 mouse embryos. The unlabelled cells were run through the Celula mvs360b chip with the maximum sorting speed and the laser turned on. Afterwards, the cells were transplanted into sublethally irradiated Ly5.1/Ly5.1 recipient mice at the dose 1 e.e. per recipient. Unmanipulated mouse AGM region cells were used as a control. No difference in HSC activity between the experimental and control groups was found (data not shown). This indirectly suggested that the on-chip microfluidic FACS did not functionally affect human AGM region HSCs. Thus, the loss of HSC activity due to immunolabelling should also be considered.

3.3.7. Immunophenotype of Human AGM Region HSCs

Since the on-chip microfluidic cell sorting approach was not successful in establishing the immunophenotype of human AGM region HSCs, the magnetic cell separation was employed as an alternative approach. To this end, AGM region cells from CS 15–17 human embryos were sorted based either on CD45 or CD144

expression using magnetic beads. Antigen-positive and negative cell populations were isolated and individually transplanted into sublethally irradiated NSG mice. In two out of three independent experiments performed with anti-human CD45 magnetic beads, HSC activity was detected exclusively within the $CD45^+$ cell population (Figure 3.3.4). As for cell separation based on the expression of CD144, in all six experiments HSCs could be detected neither within $CD144^+$ nor $CD144^-$ cell population. More experiments need to be performed to obtain more stringent statistical evidence, but the above observations suggested that anti-CD144 antibody may functionally impair human AGM region HSC. Interestingly, the anti-CD144 antibody used in the present study (clone TEA 1/31) has been shown to possess a moderate CD144 blocking activity, as assessed *in vitro* by changes in paracellular permeability of human umbilical vein endothelial cells (Corada *et al.*, 2001).

Due to a limited availability of human embryonic tissues, it was not feasible to estimate the effect of CD144 blockade on human HSC function in a systematic experiment. Therefore, mouse AGM region HSCs were used to test the hypothesis that CD144 blocking antibodies attenuated HSC activity. To this end, cell suspensions prepared from AGM regions from day 11.5 Ly5.2/Ly5.2 mouse embryos were labelled with either anti-mouse CD144, CD45 or both CD144 and CD45 monoclonal antibodies and individually transplanted into sublethally irradiated Ly5.1/Ly5.1 recipient mice at the dose 1 e.e. per recipient. Unlabelled mouse AGM region cells were transplanted to serve as a positive control. In total, three independent experiments were performed. The anti-mouse CD144 antibody used in

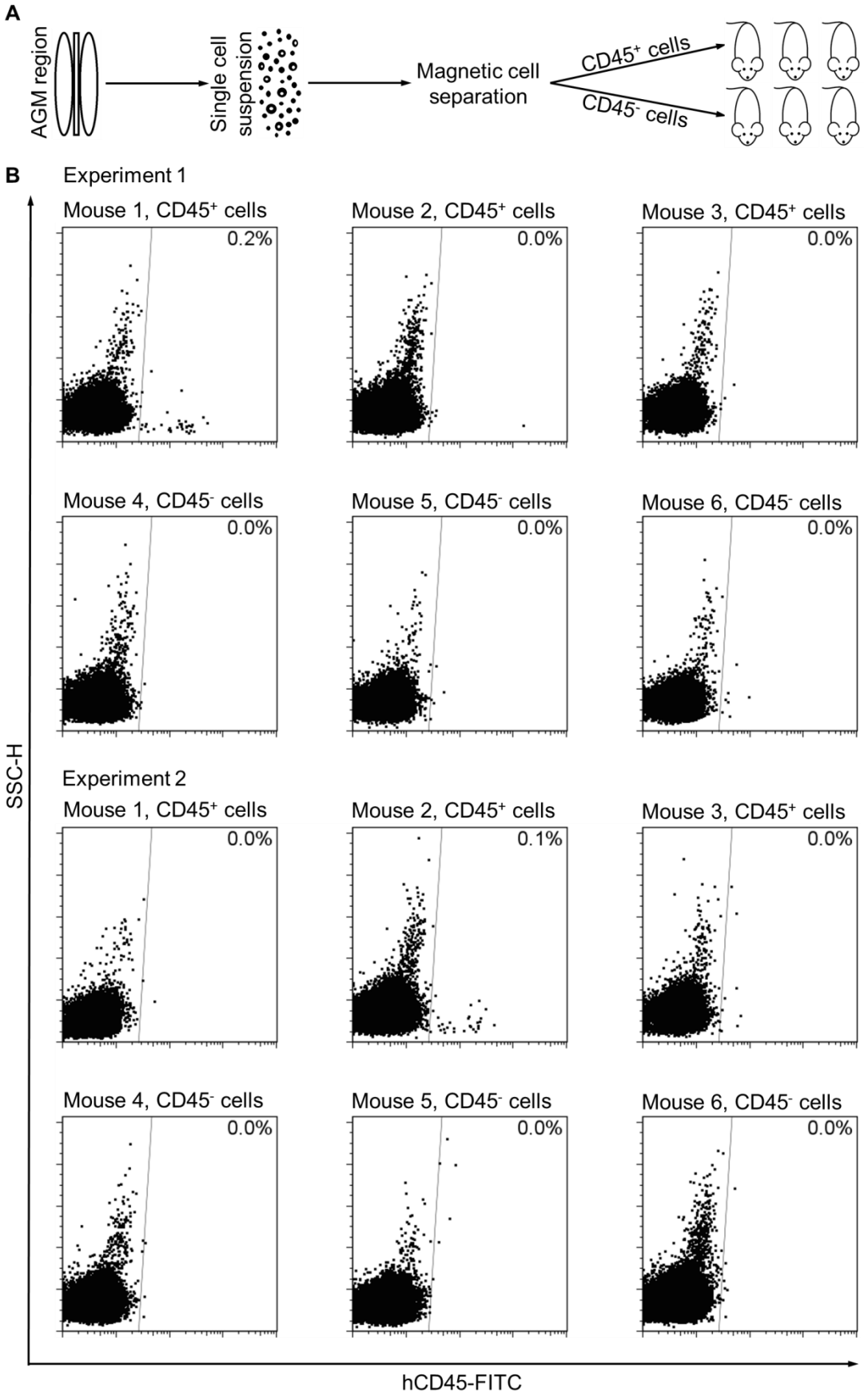

A
AGM region
Single cell suspension
Magnetic cell separation
CD45+ cells
CD45- cells
B
Experiment 1
Mouse 1, CD45+ cells
0.2%
Mouse 2, CD45+ cells
0.0%
Mouse 3, CD45+ cells
0.0%
Mouse 4, CD45- cells
0.0%
Mouse 5, CD45- cells
0.0%
Mouse 6, CD45- cells
0.0%
Experiment 2
Mouse 1, CD45+ cells
0.0%
Mouse 2, CD45+ cells
0.1%
Mouse 3, CD45+ cells
0.0%
Mouse 4, CD45- cells
0.0%
Mouse 5, CD45- cells
0.0%
Mouse 6, CD45- cells
0.0%
SSC-H
hCD45-FITC

Figure 3.3.4. Xenotransplantation experiments revealed that human AGM region HSCs are CD45$^+$. (A) AGM region cells from two CS 17 human embryos were immunolabelled with anti-human CD45 magnetic beads and separated into CD45$^+$ and CD45$^-$ cell populations, each of which was individually transplanted into sublethally irradiated NSG recipient mice. **(B)** Representative flow cytometry plots show the presence of human CD45$^+$ haematopoietic cells in the peripheral blood of two NSG recipient mice from two independent experiments (mouse 1 from experiment 1 and mouse 2 from experiment 2). Each mouse was transplanted with 0.33 e.e. of CD45$^+$ or CD45$^-$ human AGM region cells. Peripheral blood flow cytometry analysis was performed 2 months after transplantation.

these experiments (clone 11D4.1) was a blocking antibody (Gotsch *et al.*, 1997). Assessing recipient mouse peripheral blood 2 months after transplantation, it was found that in the experimental variants where mouse AGM region cells had been treated with the anti-CD144 blocking antibody alone or in a combination with an anti-CD45 antibody, two out of eight and two out of nine recipients, respectively, were found engrafted with donor HSCs. For comparison, in the experimental variant where cells had been exposed to an anti-CD45 antibody alone and in the control variant, five out of nine and five out of eight recipients, respectively, were found repopulated with donor haematopoietic cells (Figure 3.3.5). From the above data, it was concluded that CD144 blockade caused considerable functional impairment of mouse AGM region HSCs. It is very likely that CD144 blockade was a reason why human AGM region HSCs could not be detected in the long-term repopulation assay following the cell purification based on the expression of CD144 antigen using both the on-chip microfluidic FACS and the magnetic cell separation.

Although it has not formally been shown that human AGM region HSCs are $CD144^{+}$, several pieces of indirect evidence suggest that the first human HSCs do indeed express CD144. Firstly, sorted human AGM region HSCs could be detected neither within $CD144^{+}$ nor $CD144^{-}$ cell population. The finding that the blockade of CD144 considerably attenuates HSC activity hints that human AGM region HSCs reside within the $CD144^{+}$ cell population with a high probability. Secondly, the vast majority of human AGM region $CD144^{+}CD45^{+}$ cells reside in the AoV, which has been shown to possess the entire human AGM region HSC activity. Thirdly, human AGM region $CD144^{+}CD45^{+}$ cells express a number of antigens expressed by human

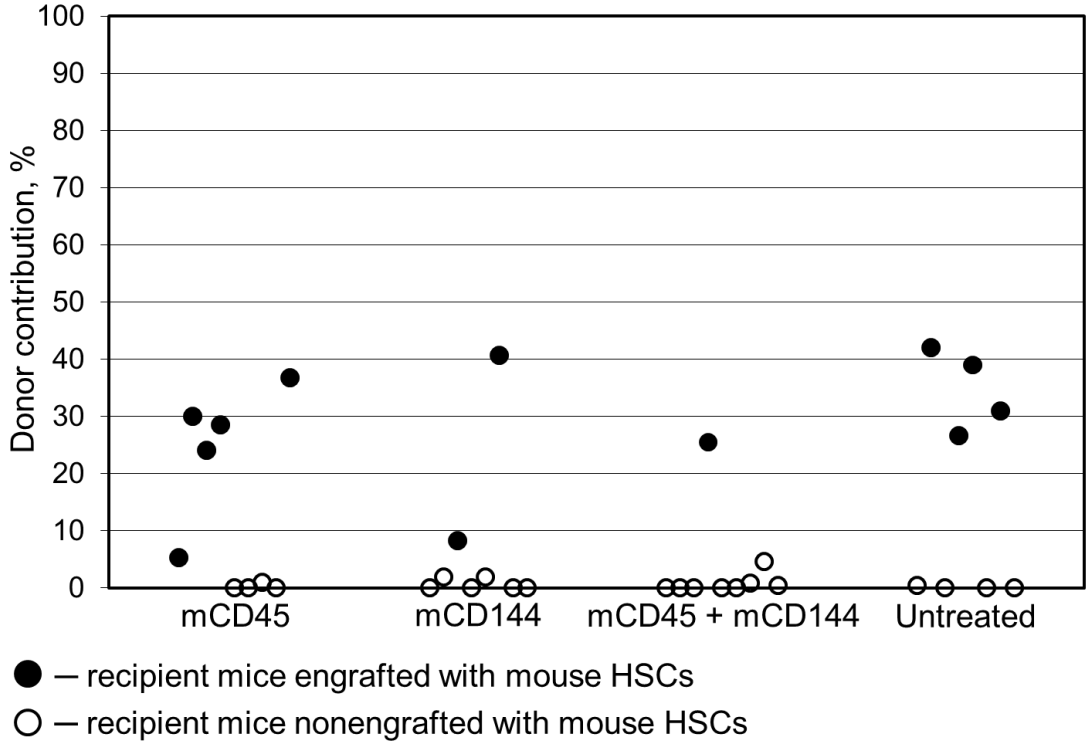


Figure 3.3.5. Anti-mouse CD144 blocking monoclonal antibody attenuates HSC activity of mouse AGM region cells. A single cell-suspension was prepared from day 11.5 Ly5.2/Ly5.2 mouse AGM regions and split into four equal parts. Each of the three parts of the cell suspension was incubated for 30 min with either anti-mouse CD144, CD45 or both CD144 and CD45 monoclonal antibodies, washed and individually transplanted into sublethally irradiated Ly5.1/Ly5.1 recipient mice. One part of the cell suspension was left unmanipulated and transplanted as a control. Recipient peripheral blood was assessed for the presence of Ly5.2/Ly5.2 $CD45^+$ cells by flow cytometry 2 months after transplantation. In all cases, cell dose transplanted was 1 e.e. per recipient. Three independent experiments were performed.

adult HSCs, for example, CD34, c-Kit, Thy-1 and CD105 (Figure 3.3.6). Fourthly, the $CD144^{+}CD45^{+}$ cell population contains almost all human AGM region CFU-Cs. In four independent experiments, human AGM region cells were immunolabelled and sorted into four cell populations based on the expression of CD144 and CD45 (Figure 3.3.7 A). Each of the sorted populations was individually plated into the methylcellulose medium supplemented with human cytokines. After 14 days of culture, human haematopoietic colonies were enumerated. The average number of CFU-Cs in the $CD144^{-}CD45^{-}$, the $CD144^{+}CD45^{-}$, the $CD144^{+}CD45^{+}$ and the $CD144^{-}CD45^{+}$ cell populations was 0.3±0.5, 3.8±3.0, 89.5±49.9 and 17.8±21.5 CFU-Cs per e.e., respectively. As assessed with the Paired-Samples T Test, the $CD144^{+}CD45^{+}$ cell population contained statistically significantly more CFU-Cs than each of the other three sorted cells populations ($P<0.05$) (Figure 3.3.7 B and Tables 3.3.1 and 3.3.2). To make the data more representative, the normalisation against the total number of CFU-Cs per e.e. was performed for each individual human embryo used in the CFU-C immunophenotyping experiments. This revealed that the $CD144^{+}CD45^{+}$ cell populations contained 83±7.8% of total human AGM region CFU-Cs. Taken together all the above, it can be claimed with a high confidence that human AGM region HSCs are $CD144^{+}CD45^{+}CD34^{+}$c-$Kit^{+}$Thy-$1^{+}CD105^{+}$.

3.3.8. Discussion

In this result subchapter, it has been shown that there exists the dorso-ventral polarity in haematopoietic activity within the human embryonic dorsal aorta. In the human embryo, the AoV contains statistically significantly more $CD45^{+}$ cells, cells with HSC immunophenotype and CFU-Cs than the AoD. More importantly, the AoV

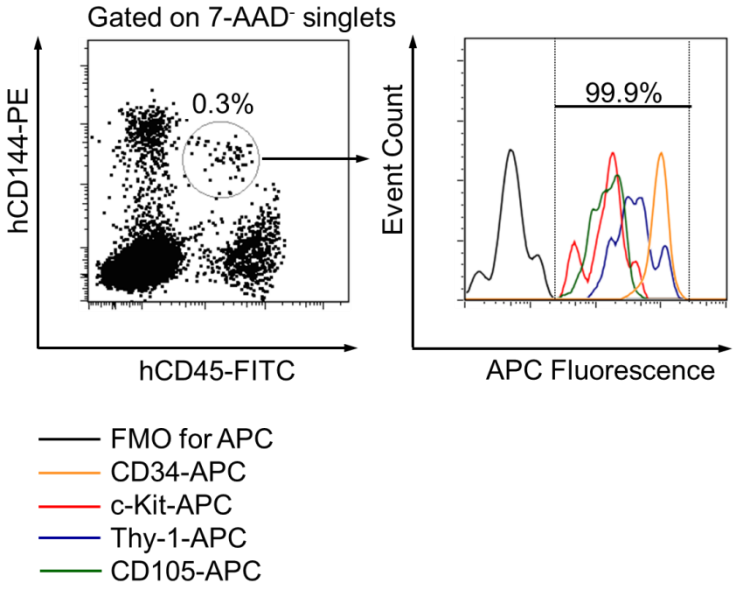


Figure 3.3.6. Expression of CD34, c-Kit, Thy-1 and CD105 by CD144^{+}CD45^{+} cells from the human AGM region. Human AGM region cells from one human embryo were split into several parts and immunolabelled with anti-human CD144-PE, CD45-FITC and either CD34, c-Kit, Thy-1 or CD105 APC-conjugated monoclonal antibodies. Based on the FMO control for APC, the expression of the later four markers was assessed in gated CD144^{+}CD45^{+} cells. At least two independent experiments were performed for each antigen.

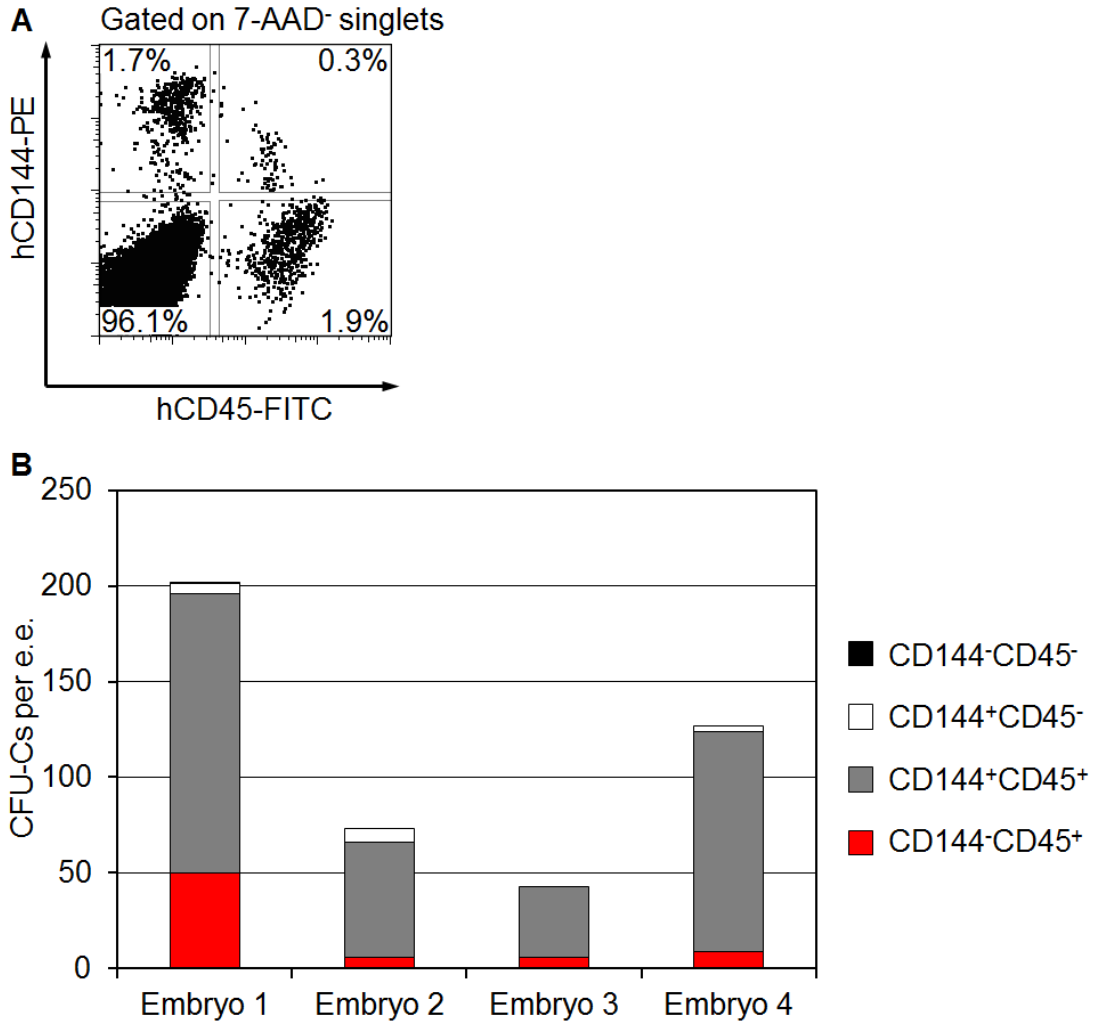


Figure 3.3.7. Immunophenotype of human AGM region CFU-Cs. (A) Human AGM region cells from four human embryos were sorted into four cell populations based on the expression of CD144 and CD45 antigens. **(B)** Each population was individually plated into methylcellulose medium supplemented with human cytokines, and after 14 days human haematopoietic colonies were enumerated microscopically.

Population	Embryo 1	Embryo 2	Embryo 3	Embryo 4
	Absolute live cell number obtained per e.e.			
$CD144^{-}CD45^{-}$	42087	91412	80572	213971
$CD144^{+}CD45^{-}$	4533	4303	5924	5860
$CD144^{+}CD45^{+}$	1484	801	869	1490
$CD144^{-}CD45^{+}$	3950	1541	4208	3611

Table 3.3.1. Absolute live cell numbers obtained per e.e. sorting cells from four different human AGM regions into $CD144^{-}CD45^{-}$, $CD144^{+}CD45^{-}$, $CD144^{+}CD45^{+}$ and $CD144^{-}CD45^{+}$ cells populations.

Population	Embryo 1	Embryo 2	Embryo 3	Embryo 4
	CFU-Cs frequency, %			
$CD144^{-}CD45^{-}$	0	0	0	0
$CD144^{+}CD45^{-}$	0.11	0.16	0	0.05
$CD144^{+}CD45^{+}$	9.8	7.5	4.3	7.7
$CD144^{-}CD45^{+}$	1.3	0.39	0.14	0.25

Table 3.3.2. CFU-C frequency within $CD144^{-}CD45^{-}$, $CD144^{+}CD45^{-}$, $CD144^{+}CD45^{+}$ and $CD144^{-}CD45^{+}$ cell populations sorted from four different human AGM regions.

possesses the entire human AGM region HSC activity. Thus, it is reasonable to assume that the AoV contains the niches in which the first human HSCs emerge. In future, the identification and characterisation of such niches would facilitate our understanding of the key factors required for the generation of adult-type HSCs. This knowledge would not only be of academic interest but also of enormous practical value in the development of the methods for *in vitro* differentiation of human pluripotent stem cells into transplantable HSCs.

According to current views, it is possible that intra-aortic cell clusters budding from the ventral wall of the embryonic dorsal aorta and underlying mesenchyme are constituted from the mentioned above HSC niches (Jaffredo et al., 1998; Taoudi et al., 2008; Yokomizo and Dzierzak, 2010). Due to a number of reasons discussed below, one should not underestimate other locations for HSC niches within the AoV. Firstly, in the human, formation of $CD34^{+}CD45^{+}$ cell clusters occurs between CS 12 and 16 (Tavian et al., 1996; Tavian et al., 1999). In section 3.2.3, it has been shown that one human AGM region contains between one and three HSCs; however, the number of intra-aortic cell clusters is much higher (Tavian et al., 1996; Tavian et al., 1999), suggesting that not every cluster contains HSCs. Secondly, although HSCs are still detected at CS 17, intra-aortic cell clusters are reported to disappear by that stage (Tavian et al., 1996; Tavian et al., 1999), indicating that the link between HSCs and haematopoietic cell clusters needs to be elucidated further. Thirdly, it has been shown that the maturation of definitive HSCs in the mouse embryo also occurs underneath the luminal layer of the dorsal aorta (Rybtsov *et al.*, 2011). Taking together all the above, the identification of exact HSC location will be a rather

challenging task. One of the possible initial approaches to this scientific question would be a detailed characterisation *in situ* of the cells surrounding human AGM region HSCs. For this, the first requirement is to establish a precise immunophenotype which would allow separating the earliest human HSCs and more differentiated haematopoietic progenitors present in the AGM region.

If one compares the dorso-ventral polarity in haematopoietic activity within the human versus the mouse dorsal aorta, it should be noted that in the day 11.5 mouse embryo there only exists the polarity in pre-HSC and HSC activity. Mouse mature haematopoietic cells and CFU-Cs are uniformly distributed in the wall of the dorsal aorta (Taoudi and Medvinsky, 2007). In contrast, in the human embryo, the dorso-ventral polarity in haematopoietic activity within the dorsal aorta is absolute. Therefore, studying the differential gene expression in the AoV versus the AoD can identify potential factors regulating the emergence of HSCs, and it is possible that a result obtained by using human but not mouse embryonic tissues will be 'cleaner'. This, of course, does not mean that the data obtained for the human embryo should be blindly and fully extended to the mouse embryo since there is a possibility that the mechanism regulating the formation of definitive HSCs in the human and the mouse are indeed different.

The preliminary long-term repopulation assay data on the immunophenotype of human AGM region HSCs presented in this result subchapter strongly suggest that the earliest human HSCs are $CD144^{+}CD45^{+}$. Remarkably, HSCs from the mouse AGM region are also $CD144^{+}CD45^{+}$ (Taoudi *et al.*, 2005). Using flow cytometry

analysis, it has also been found that the $CD144^{+}CD45^{+}$ cell population from the human AGM region is entirely positive for CD34, c-Kit and Thy-1, which all are markers of human adult HSCs. Thus, the immunophenotype of human AGM region HSCs can be extended further without a need to perform transplantation experiments. Unfortunately, this does not help much in addressing the two important biological questions raised in the present thesis. The first question is related to the identification and characterisation of HSC niches in the AoV. The second question touches on the mechanism underlying the high regenerative potential of early human embryonic HSCs. To answer both of these questions, it is crucial to separate human AGM region HSCs and more differentiated haematopoietic progenitors. As it has been shown above, the $CD144^{+}CD45^{+}$ cell population from the human AGM region contains both HSCs and CFU-Cs. Unluckily, it was not possible to separate these two haematopoietic cell populations by applying additional human HSC markers since virtually all $CD144^{+}CD45^{+}$ cells from the human AGM region also expressed CD34, c-Kit, Thy-1 and CD105. Currently, this is the aim of my ongoing work to find ways how to separate human AGM region HSCs and CFU-Cs.

4. SUMMARY AND PERSPECTIVES

Prior to this study, nothing was known about the development of haematopoietic stem cells in the human embryo. Medvinsky and Dzierzak (1996) have shown that the AGM region is the prime site for the generation of HSCs in the mouse embryo. I tested whether this could be extended to the human embryo. Since HSCs can be assessed only by transplantation into preconditioned recipients, the long-term repopulation assay was rigorously employed in the present study. Transplanting sublethally irradiated NSG mice with single cell suspensions prepared from various intra- and extra-embryonic tissues obtained from human embryos at different developmental stages, the spatio-temporal mapping of HSC activity within the human embryo has been performed. It has been found that, similar to the mouse embryo (Muller *et al.*, 1994; Taoudi *et al.*, 2005), the earliest human embryonic HSCs are detected in the AGM region (around week 5 of development), specifically in the ventral wall of the dorsal aorta. Approximately one week later, the human yolk sac and embryonic liver also acquire HSC activity. In contrast to the mouse embryo (de Bruijn *et al.*, 2000; Gekas *et al.*, 2005; Ottersbach and Dzierzak, 2005), umbilical cords and placentas from early human embryos do not contain HSCs. It was reported by others that the human placenta acquires true HSC activity around week 9 of development (Robin *et al.*, 2009). Thus, although the development of HSCs is mainly conserved between the mouse and the human embryo, there are a number of substantial differences between the two species.

Although a key role of the AGM region in the production of definitive HSCs is widely acknowledged in the field of mouse embryonic haematopoiesis, there still

remains a controversy regarding the origin of the first definitive HSCs. The main reason for this is that in the mouse embryo the first definitive HSCs are detected at approximately the same time in the dorsal aorta, the yolk sac, the umbilical and vitelline arteries and the placenta (reviewed by Medvinsky *et al.*, 2011). Therefore, it is very difficult or maybe even impossible to set up experiments in a way that would provide a final answer to the question, 'In which tissue do the first mouse definitive HSCs emerge?' In the quest for the answer to this question one should consider that it is also possible that HSCs may arise independently in different intra- and extra-embryonic sites, which is equally difficult to prove. In the human embryo, the appearance of HSCs in different tissues is clearly resolved, with the AGM region being the first site where HSC are detected. Thus, while the issue of the origin of definitive HSCs is not resolved in the mouse embryo and nonmammalian vertebrate embryos, the data obtained in the experiments with human embryos shift the balance further towards the priority of the AGM region in the generation of the first HSCs. I believe that this essential result became possible due to extended time required for the development of the human embryo.

Now, it is clear that the first human HSCs emerge in the AGM region. However, this does not necessarily mean that the human definitive HSC lineage (pre-HSCs and yet undescribed earlier HSC precursors) arises in the AGM region. It is still possible that human HSC precursors emerge, for example, in the yolk sac and then migrate to the AGM region to become there the first definitive HSCs. Although I did make attempts to address the question on the existence of human pre-HSCs employing the *ex vivo* AGM region and yolk sac explant, reaggregate and coaggregate culture systems

developed in our laboratory to study mouse pre-HSCs (Rybtsov *et al.*, 2011; Taoudi *et al.*, 2008), the experiments with human embryonic tissues were not successful, and even the maintenance of HSC activity could not be reproducibly achieved (data not shown). Due to a limited availability of human embryonic tissues and an unclear approach how to address the question, these experiments have been postponed in order to put as a priority other fundamental questions on the embryonic development of human HSCs. Hopefully, the ongoing work in the laboratory, for example, the derivation of stromal cell lines from the human AGM region will provide additional tools for identifying and manipulating human pre-HSCs.

An unexpected but very important finding of this study was that human AGM region HSCs possess an unprecedented regenerative potential. Upon transplantation into sublethally irradiated NSG mice, a single HSC from the human AGM region produces around 600 daughter HSCs during a period of 5–9 months. Although it is unlikely that early human embryonic HSCs will ever be used in clinical haematology as a transplant, the above finding has potential clinical applications. Exploring the mechanisms underlying the high propagation capacity of human AGM region HSCs, it may be possible to develop methods to increase the regenerative potential of human umbilical cord blood and adult bone marrow HSCs, which are currently used in the clinic. To begin addressing this issue, we are planning to perform the whole transcriptome analysis to establish the list of genes differentially expressed between human AGM region and umbilical cord blood HSCs. For this, it is required first to establish the precise immunophenotype of the earliest human HSCs to be able to purify the cells for the gene expression analysis. In the course of the present study, it

has been established that HSCs from the human AGM region are $CD144^{+}CD45^{+}CD34^{+}$c-$Kit^{+}$Thy-$1^{+}CD105^{+}$. Although this immunophenotype provides up to 1000-fold enrichment of HSCs if compared to unseparated human AGM region cells, it does not segregate HSCs from more differentiated haematopoietic progenitors. Therefore, the aim of my current experimental work is to find cell surface markers permitting the separation of embryonic HSCs and CFU-Cs.

In parallel, we are planning to perform gene expression analysis in the human embryonic HSC niche. This may provide important information on potential key factors involved in the generation and maintenance of HSCs. I have shown that HSCs within the human AGM region reside exclusively in the AoV. In cell sorting and xenotranplantation experiments, data on the immunophenotype of early human embryonic HSCs have been obtained. This will allow me to localise cells with HSC immunophenotype within the AoV. It is reasonable to think that the cells surrounding immunophenotypic HSCs are the cells forming the early embryonic HSC niche. Based on the data of others (Tavian *et al.*, 2010), it is likely that the cells with HSC immunophenotype will be found mainly in the intra-aortic cell clusters budding from the AoV. I will first try to carefully localise intra-aortic cell clusters using the whole-mount immunostaining technique and confocal microscopy. Then, cells within and underneath the intra-aortic cell clusters and cells from the AoD (negative control) will be microdissected using the laser capture technique and will be used for the differential gene expression analysis.

In summary, this study has provided a systematic spatio-temporal picture of HSC emergence in the early human embryo and identified the AGM region as the primary source of HSCs with unprecedented self-renewal capacity. Future comparative studies of early human embryonic HSCs and their foetal, neonatal and adult counterparts may shed light on mechanisms of HSC self-renewal and aging.

5. APPENDIX A: FLOW CYTOMETRY CONTROLS

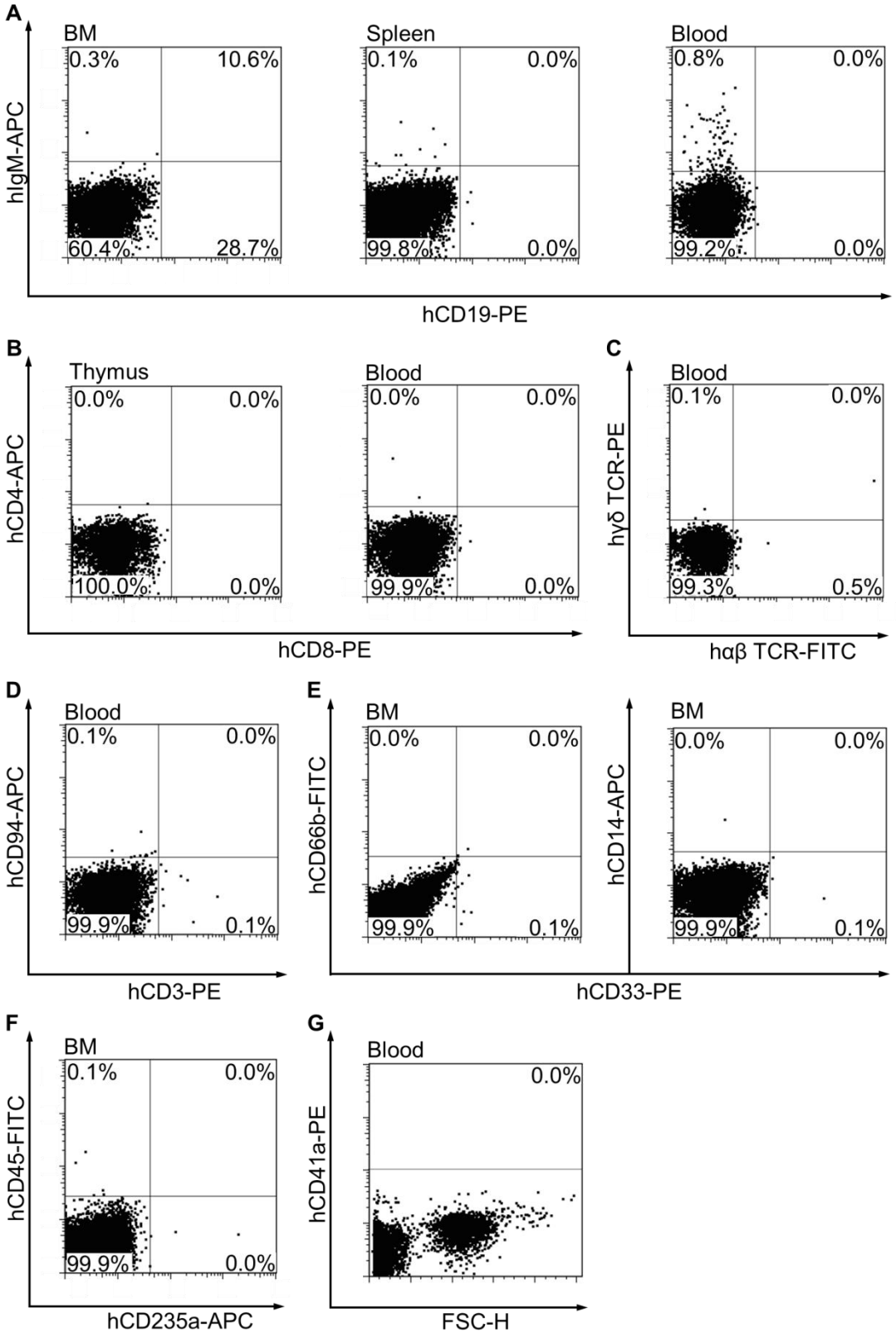
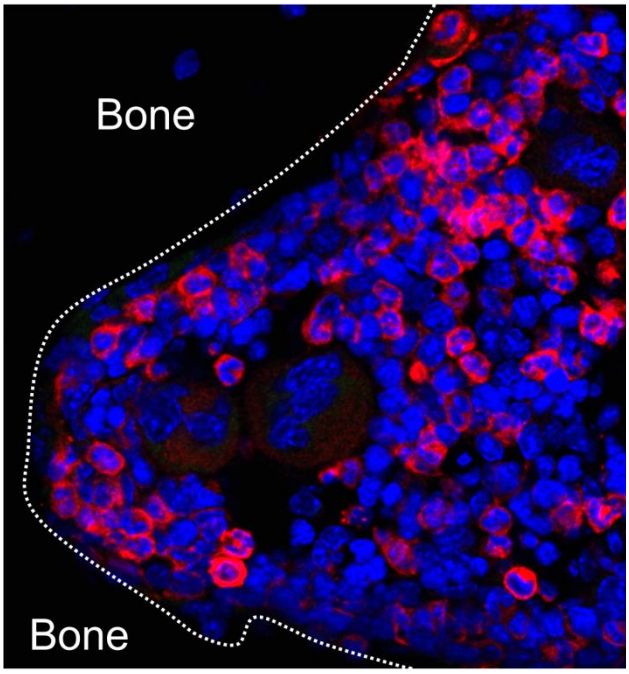


Figure A.1. Flow cytometry controls. Considering disadvantages of isotype controls (Keeney *et al.*, 1998), cell populations of interest were usually gated based on autofluorescence of cells from sublethally irradiated NSG mice injected with DPBS. The cells from such control mice were treated with antigen-specific anti-human monoclonal antibodies in parallel with the cells from mice transplanted with human cells. Representative flow cytometry plots show gates used for designation of human B cells **(A)**, T cells **(B, C)**, NK and NKT cells **(D)**, granulocytes and monocytes/macrophages **(E)**, erythroid cells **(F)** and platelets **(G)** in the peripheral blood, bone marrow, spleen and/or thymus of NSG recipient mice, as shown on Figure 3.1.4. BM, bone marrow.

6. APPENDIX B: CONFOCAL MICROSCOPY CONTROL



hCD45 FITC

mCD45 Alexa Fluor 546

DAPI

Figure B.1. Confocal microscopy control for anti-human CD45 monoclonal antibody staining. To test the specificity of anti-human CD45 monoclonal antibody, femurs from sublethally irradiated NSG mice injected with DPBS were used. After the bones were fixed, decalcified, snap-frozen and sectioned, tissues were stained with anti-human and anti-mouse CD45 monoclonal antibodies, followed by nuclear counterstaining with DAPI. Images were obtained employing confocal microscopy.

7. APPENDIX C: PUBLICATION

Ivanovs, A., Rybtsov, S., Welch, L., Anderson, R.A., Turner, M.L., and Medvinsky, A. (2011). *Highly potent human hematopoietic stem cells first emerge in the intraembryonic aorta-gonad-mesonephros region*. J Exp Med *208*, 2417-2427.

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Article

Highly potent human hematopoietic stem cells first emerge in the intraembryonic aorta-gonad-mesonephros region

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Hematopoietic stem cells (HSCs) emerge during embryogenesis and maintain hematopoiesis in the adult organism. Little is known about the embryonic development of human HSCs. We demonstrate that human HSCs emerge first in the aorta-gonad-mesonephros (AGM) region, specifically in the dorsal aorta, and only later appear in the yolk sac, liver, and placenta. AGM region cells transplanted into immunodeficient mice provide long-term high level multilineage hematopoietic repopulation. Human AGM region HSCs, although present in low numbers, exhibit a very high self-renewal potential. A single HSC derived from the AGM region generates at least 300 daughter HSCs in primary recipients, which disseminate throughout the entire recipient bone marrow and are retransplantable. These findings highlight the vast regenerative potential of the earliest human HSCs and set a new standard for in vitro generation of HSCs from pluripotent stem cells for the purpose of regenerative medicine.

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Abbreviations used: AGM region, aorta-gonad-mesonephros region; CS, Carnegie stage; e.e., embryo equivalent; HSC, hematopoietic stem cell; NSG, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/Sz; STR, short tandem repeat; UCB, umbilical cord blood.

Hematopoietic stem cells (HSCs) are multipotent stem cells that emerge early during embryogenesis and maintain hematopoiesis throughout the entire lifespan of the organism (Dzierzak and Speck, 2008; Medvinsky et al., 2011). Although in various vertebrate species the first hematopoietic differentiation occurs in the yolk sac (Moore and Metcalf, 1970), a growing body of data suggests that the aorta-gonad-mesonephros (AGM) region plays a key role in the generation of definitive HSCs (Dieterlen-Lievre, 1975; Medvinsky et al., 1993; Müller et al., 1994; Medvinsky and Dzierzak, 1996; de Bruijn et al., 2002; Zovein et al., 2008; Boisset et al., 2010), possibly through hematopoietic transition of endothelial cells of the dorsal aorta, which has been most clearly shown in zebrafish (Bertrand et al., 2010; Kissa and Herbomel, 2010). The mouse AGM region is capable of autonomous initiation and expansion of HSCs (Medvinsky and Dzierzak, 1996; Cumano et al., 2001; Taoudi et al., 2008). The early umbilical cord and the placenta are also implicated in HSC development (de Bruijn et al., 2000; Gekas et al., 2005; Ottersbach and Dzierzak, 2005; Robin et al., 2009). In the mouse embryo, the first definitive HSCs are detected at approximately the same time in different tissues (Müller et al., 1994; de Bruijn et al., 2000; Kumaravelu et al., 2002; Gekas et al., 2005; Ottersbach and Dzierzak, 2005), thus their primary origin remains debatable (Medvinsky et al., 2011).

Qualitative and quantitative assessment of HSCs can only be performed functionally using in vivo limiting dilution long-term repopulation assays (Szilvassy et al., 1990). Although human HSCs have been extensively studied in fetal, neonatal, and adult sources by transplantation into immunodeficient mice (Larochelle et al., 1996; Conneally et al., 1997; Wang et al., 1997), at early embryonic stages HSCs were assayed only in the liver (Oberlin et al., 2010) and the placenta (Robin et al., 2009). To date, hematopoiesis in the AGM region and the yolk sac has been characterized only by immunohistological methods and in vitro assays (Tavian et al., 1996, 1999, 2001; Oberlin et al., 2002).

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It is thought that HSCs mature in intraaortic cell clusters budding from the ventral wall of the dorsal aorta (Tavian et al., 1996, 1999; Jaffredo et al., 1998; Taoudi et al., 2008; Yokomizo and Dzierzak, 2010). The maturation of HSCs also occurs in deeper layers of the dorsal aorta (Rybtsov et al., 2011).

In this study, we describe spatiotemporal distribution of HSCs in the early human embryo and provide evidence that the AGM region is the first generator of highly potent HSCs in the human. Upon transplantation into immunodeficient mice, HSCs derived from the AGM region extensively propagate, migrate to different bones throughout the recipient, and provide progressively increasing long-term multilineage hematopoietic repopulation of the host animal with robust potential for retransplantation. Our study reveals that the first human HSCs possess an unprecedented self-renewal capacity. A better understanding of embryonic development of human HSCs may be instrumental for creating clinically relevant protocols for the production of HSCs from human embryonic and induced pluripotent stem cells (Kaufman, 2009).

RESULTS

Spatiotemporal distribution of HSCs in the early human embryo

AGM regions, yolk sacs, livers, umbilical cords, and placentas were obtained from human embryos between Carnegie stages (CSs) 12 and 17 (O'Rahilly and Müller, 1987). Cell suspensions prepared from these tissues were transplanted into irradiated NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/Sz (NSG) mice (Shultz et al., 2005) within 3–4 h of termination of pregnancy. Cells from each individual tissue were split equally between two to four NSG recipient mice. In 27 independent experiments, HSCs were detected in 10 AGM regions and 3 yolk sacs by their capacity to provide human long-term (4–8 mo) multilineage hematopoietic repopulation and by their retransplantability into secondary recipients (Table I). In CS 14 and 15 human embryos, HSCs were detected exclusively in the AGM region (5 embryos). At CS 16 and 17, HSCs were found either solely in the AGM region (2 embryos) or both in the AGM region and the yolk sac (3 embryos). Thus, HSCs appear in the human AGM region at least 5 d earlier than in the yolk sac (Table I).

Out of 26 transplanted livers, none contained HSCs; however, two provided unilineage T cell engraftment (Table I and not depicted). In one case, exclusive T cell engraftment was also observed in a recipient of an AGM region (Table I and not depicted). In these three cases, human T cell contribution increased during the first 4–6 mo, and then decreased on a similar timescale (not depicted). Umbilical cords and placentas contained no HSCs at all stages tested (Table I).

Short tandem repeat (STR) analysis (Cotton et al., 2000) confirmed that the human cells detected in NSG recipient mice repopulated by AGM region, yolk sac, and liver cells originated from the donor embryo, with no evidence of contamination by maternal cells (Tables S1–S4).

The dorsal aorta is the source of HSC activity within the human AGM region

In the mouse embryo, definitive HSCs first appear in the dorsal aorta (de Bruijn et al., 2000; Taoudi and Medvinsky, 2007). We tested whether this is true for the human embryo. For these experiments, we used human embryos between CS 15 and 17. The human AGM region was subdissected into the dorsal aorta and the urogenital ridges, as previously described for the mouse AGM region (de Bruijn et al., 2000; Taoudi and Medvinsky, 2007). Cell suspensions prepared from these tissues were transplanted into irradiated NSG recipient mice. In 10 independent experiments performed, HSCs were detected in six dorsal aortas, but never in the urogenital ridges (see Materials and methods).

High-level hematopoietic repopulation by early embryonic HSCs

In NSG mice engrafted with human HSCs from the AGM region and the yolk sac, donor CD45$^+$ hematopoietic cells first became detectable in the peripheral blood of recipients 1.5–3 mo after transplantation, with donor cell levels reaching

Table I. The first HSCs in the human embryo emerge in the AGM region

Carnegie stage (days)	Embryonic tissues transplanted				
	AGM region	Yolk sac	Liver	Umbilical cord	Placentas
12 (26)	0/2	0/2	0/2	NA	1[c]/2
13 (28)	0/2	NA	0/1	NA	1[c]/2
14 (32)	1[b]/7	0/7	0/7	0/4	2[c]/7
15 (33)	4[a]/5	0/3	0/5	0/4	1[c]/2
16 (37)	2[a] + 1[b]/6	1[a]/4	0/6	0/6	1[c]/3
17 (41)	3[a]/5	2[a]/4	2[b]/5	0/3	1[c]/3
Total	10[a] + 1[b]/27	3[a]/20	2[b]/26	0/17	7[c]/19

Cell suspensions prepared from AGM regions, yolk sacs, livers, umbilical cords, and placenta obtained from CS 12–17 human embryos were individually transplanted into irradiated NSG mice. Data shown are the number of tissues that gave human hematopoietic repopulation compared to the total number of tissues transplanted. The numbers in parentheses indicate an approximate postovulatory gestational age in days accepted for each CS (O'Rahilly and Müller, 1987). NA, not assessed.

[a]Long-term embryonic multilineage repopulation.

[b]Long-term embryonic unilineage T cell repopulation.

[c]Transient maternal unilineage T cell repopulation.

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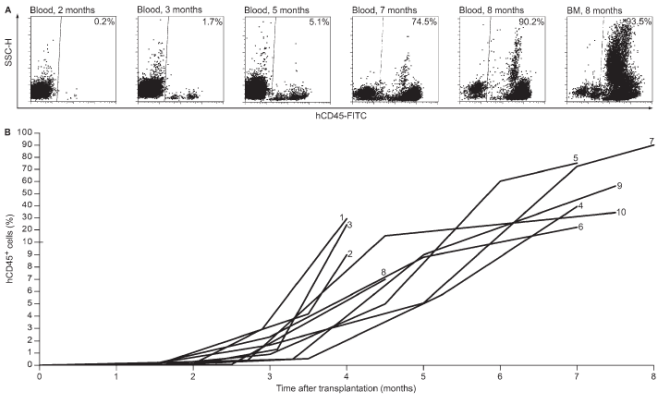

Figure 1. Human AGM region–derived HSCs provide progressive growth of hematopoietic contribution in NSG mice. (A) Representative flow cytometry plots show hematopoietic cells in the peripheral blood and BM of an NSG mouse transplanted with 0.33 e.e. of AGM region cells. In this case, the AGM region was obtained from a CS 16 embryo. The repopulation kinetics was monitored for 8 mo. hCD45, human CD45. (B) Human hematopoietic engraftment kinetics in the peripheral blood of 10 NSG recipient mice transplanted with human AGM region or yolk sac cells. The numbers at the end of the time series correspond to the recipient identification numbers in Table II.

~0.2% on average (range, 0.06–0.54%; $n = 13$) of total blood leukocytes. In all except one case, the percentage of human leukocytes in the peripheral blood progressively increased and reached up to 90% of total blood leukocytes by 8 mo after transplantation (Fig. 1 and Table II). By the end of the observation period (4–8 mo), on average ~40% (range, 7–90%; $n = 10$) of leukocytes in the recipient peripheral blood were of human origin (Fig. 1 and Table II). In the BM, the percentage of human $CD45^+$ cells was higher than in peripheral blood, reaching on average ~70% (range, 42–94%; $n = 7$) of total BM leukocytes (Table II). Previous studies have shown that transplantation of many human umbilical cord blood (UCB) or adult BM HSCs into immunodeficient mice results in blood chimerism that is <50% at 10 wk and frequently decreases after several months (Cashman et al., 1997; Ishikawa et al., 2005; Liu et al., 2010). However, recent experiments have shown that 10–20 UCB HSCs per one NSG recipient mouse are able to provide up to 40% repopulation in the peripheral blood by month 8 after transplantation (Notta et al., 2011).

Multilineage differentiation potential of early embryonic HSCs

Human multilineage hematopoietic engraftment with AGM region and yolk sac cells was biased toward lymphopoiesis, dominated by B cells ($CD19^+$; Table III). Approximately one-third of human B cells in the recipient BM passed the pre–B cell stage, as indicated by the IgM expression on the cell surface. In the spleen, the proportion of human $CD19^+IgM^+$ cells was twice as high as in the BM, and in the peripheral blood almost all human B cells were IgM^+, replicating normal B cell development (Fig. 2 A). Human T cells ($CD3^+$) were also represented. Double-positive $CD4^+CD8^+$ cells prevailed in the thymus, and the peripheral blood contained mainly single-positive $CD4^+CD8^-$ and $CD8^+CD4^-$ T cells, which is indicative of normal intrathymic maturation of human T cells (Fig. 2 B and Table III). Almost all human T cells in the peripheral blood of recipient animals expressed αβ TCRs, as is observed in human peripheral blood. Only ~1% of T cells

Table II. High level hematopoietic repopulation by a single early embryonic HSC

Recipient no.	Tissues transplanted	Time after transplantation	Human $CD45^+$ cells in the recipient blood	Human $CD45^+$ cells in the recipient BM	Human $CD45^+$ cells in the recipient spleen
		mo	%	%	%
1	AGM region	4	29	42	4
2	AGM region	4	9	NA	NA
3	AGM region	4	24	45	31
4	AGM region	7	39	86	87
5	AGM region	7	73	93	93
6	AGM region	7	22	71	75
7	AGM region	8	90	94	96
8	Yolk sac	4.5	7	NA	NA
9	Yolk sac	7.5	56	NA	NA
10	Yolk sac	7.5	34	79	87

NSG mice transplanted with HSCs derived from the human AGM region or yolk sac were observed for 4–8 mo. At the end of each experiment, the percentage of human $CD45^+$ cells in the recipient peripheral blood, BM, and spleen was assessed by flow cytometry. NA, not assessed as the recipients died.

expressed γδ TCRs (Fig. 2 C). Additionally, human AGM region– and yolk sac–derived HSCs produced $CD94^+CD3^-$ NK cells and $CD94^+CD3^+$ NKT cells at low frequencies (Fig. 2 D and Table III).

The percentage of human $CD33^+$ myeloid cells in the recipient peripheral blood and spleen was ∼20–25 times lower than that of human lymphoid cells; however, the recipient BM contained on average 30% (range, 13–70; $n = 7$) human granulocytes ($CD33^+CD66b^+$) and monocytes ($CD33^+CD14^+$; Fig. 2 E and Table III). Megakaryocytic lineage was also readily detectable by appearance of human FSC-$H^{low}CD41a^+$ platelets in the recipient peripheral blood (Fig. 2 F). The recipient BM contained ∼8% human erythroid cells ($CD235a^+$), of which one third coexpressed CD45, indicating their immature state (Fig. 2 G).

Additionally, the BM of engrafted recipients plated into methylcellulose medium supplemented with human cytokines gave rise to human erythroid colonies (burst-forming units-erythroid [BFU-E]; Fig. 2 H and Table IV), myeloid colonies (colony-forming units-myeloid [CFU-Myeloid]; Table IV), and mixed erythromyeloid colonies (CFU-Mix; Fig. 2 I and Table IV). The human origin of hematopoietic colonies was confirmed by flow cytometry analysis (not depicted).

Early embryonic HSCs can be efficiently retransplanted

We tested whether HSCs derived from the human AGM region and yolk sac can be serially transplanted. In all eight experiments with the AGM region and two experiments with the yolk sac, transplantation of BM from primary recipients into secondary recipients resulted in sustainable human long-term multilineage hematopoietic engraftment (Fig. 3). In contrast, secondary BM transplantations from all three recipients exhibiting long-term unilineage T cell repopulation were unsuccessful. This indicates that HSCs colonize the human embryonic liver after CS 17. T cell–restricted repopulation may originate either from previously undescribed long-term persisting embryonic T cell progenitors or from human counterparts of mouse lymphopoiesis-biased γ and δ subtypes of long-term repopulating hematopoietic cells, which show no capacity for retransplantion (Dykstra et al., 2007).

Extensive amplification of early embryonic HSCs in primary recipients

Because one HSC can efficiently restore hematopoiesis in vivo (Osawa et al., 1996; Notta et al., 2011), the frequency of HSCs can be estimated using a limiting dilution assay (Szilvassy et al., 1990). In 9 out of 10 experiments transplanting human AGM region cells into two to four NSG mice, one recipient per experiment was found engrafted with human hematopoietic cells. Both recipients were repopulated in only one experiment (not depicted). To estimate HSC numbers, we used software for small numbers of replicates and nonlinear situations (Hu and Smyth, 2009). Our data fit the single-hit Poisson model ($\chi^2 = 0.108$; $P = 0.742$), demonstrating consistency between all 10 experiments performed with the AGM regions containing HSCs. The model calculations showed that one human AGM region contains ∼1.9 HSCs (95% CI, 1.0–3.3; Fig. 4 A). If all 23 CS 14–17 AGM regions are considered (including nonrepopulating ones), the frequency of HSCs is ∼0.8 per one human AGM region, which is close to the number of definitive HSCs in the mouse AGM region (Kumaravelu et al., 2002).

Statistically, human hematopoietic repopulation in each engrafted NSG mouse must have arisen mainly from one human HSC. The progressive growth in levels of repopulation with human hematopoietic cells (Fig. 1) may either be the result of gradual enhancement of productivity of this one HSC or, alternatively, the result of amplification of HSC numbers in recipients. To address this issue, the BM of engrafted primary recipients was harvested 4–8 mo after transplantation in five independent experiments. BM from an individual recipient was transferred into 6–15 secondary recipients. In these experiments, 41 out of 44 secondary recipients transplanted were found engrafted with human HSCs. Because each primary

Table III. Multilineage hematopoietic engraftment in primary recipient mice

Recipient no.	Blood				BM				Spleen				Thymus			
	B	T	NK	Myeloid	B	T	NK	Myeloid	B	T	NK	Myeloid	$CD4^-CD8^-$	$CD4^+CD8^+$	$CD4^+CD8^-$	$CD8^+CD4^-$
	% in gated human $CD45^+$ cells															
1	44	39	NA	11	48	19	NA	30	78	12	NA	9	NA	NA	NA	NA
2	67	23	3	4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
3	19	65	5	11	16	42	1	38	29	56	1	15	1	68	25	6
4	77	5	4	12	51	0.1	0.2	45	87	1	1	9	2	84	10	4
5	75	19	2	4	62	1	0.3	37	90	7	0.7	2	3	80	13	4
6	56	25	9	9	61	19	1	20	82	4	3	14	2	85	9	4
7	17	36	31	18	20	4	1.5	74	41	39	11	11	NA	NA	NA	NA
8	59	22	NA	15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	63	19	NA	14	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
10	26	60	9	6	42	12	2	43	46	41	7	7	2	69	22	7

Data shown are the percentage of B ($CD19^+$), T ($CD3^+$ or $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD8^+CD4^-$), NK ($CD94^+CD3^-$), and myeloid cells ($CD33^+$ or $CD13^+$) in the human $CD45^+$ cell population in the peripheral blood, BM, spleen, and thymus of NSG mice transplanted with AGM region and yolk sac cells. The recipient identification numbers are the same as in Table II. NA, not assessed.

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recipient was engrafted with approximately one human HSC, these results suggest that human HSCs propagated in primary recipients.

We considered an alternative possibility that the human AGM region contained some HSCs that remained dormant in primary recipients but could be activated upon secondary transplantations. To test this, we selected nonrepopulated primary recipients (Fig. 5 A, mice a and b) from five independent experiments in which at least one recipient was engrafted with AGM region cells (Fig. 5 A, mouse c). The entire BM from two coxal bones, two femurs, and two tibiae was collected from these nonrepopulated primary recipients and transferred into secondary recipients. None of secondary recipients showed human hematopoietic engraftment, confirming that the AGM region contained only one or two HSCs. Secondary transplantations of BM from nonengrafted recipients transplanted with yolk sac, liver, umbilical cord, and placental cells were also unsuccessful (two to five independent experiments were performed for each tissue).

To estimate the number of daughter HSCs generated in primary recipients by a single HSC derived from the AGM region, we performed two independent secondary transplantation experiments using the limiting dilution assay. The BM from successfully engrafted primary recipients was harvested from the sternum, humeri, ulnae, radii, coxal bones, femurs, and tibiae, and then pooled and transplanted in serial dilutions into secondary recipients. In experiment 1, the lowest dose transplanted per secondary recipient was 1/75 of total BM from primary recipient. All secondary recipients showed human long-term multilineage hematopoietic engraftment. In experiment 2, the transplanted dose was decreased further to 1/300 of total BM per recipient. Again, all secondary recipients showed human long-term multilineage hematopoietic repopulation (Fig. 4 B). However, in NSG mice transplanted with the lowest BM dose the level of human contribution, as determined in the peripheral blood 2 mo after transplantation, was <3%. Although we did not reach the limiting dose, we were close to the extinction of HSC activity. Human HSCs were also detected in spleens of primary recipients (Fig. 4 C). Thus, one HSC from the human AGM region is capable of generating at least 300 daughter HSCs upon transplantation. Previous studies have demonstrated a

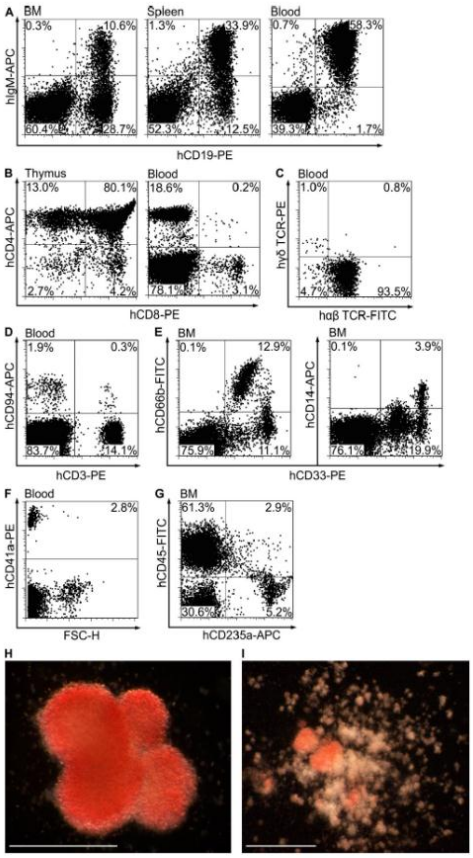

Figure 2. Human AGM region–derived HSCs provide long-term multilineage hematopoietic engraftment. Representative flow cytometry plots show human B cells (A), T cells (B and C), NK and NKT cells (D), granulocytes and monocytes (E), platelets (F), and erythroid cells (G) in the peripheral blood, BM, spleen, and/or thymus of an NSG mouse 7 mo after it was transplanted with 0.5 e.e. of AGM region cells. In this case, the AGM region was obtained from a CS 17 embryo. Note that the staining for CD4 and CD8 is shown in gated human CD45+ cells, and for TCRs in gated human CD3+ cells. Plating BM of the same recipient into methylcellulose medium supplemented with human cytokines resulted in the formation of human erythroid colonies (BFU-Es; H) and mixed erythromyeloid colonies (CFU-Mix; I). Bars, 0.5 mm.

Table IV. Human CFU-C frequency in the BM of primary recipient mice

Recipient no.	Human CFU-C no. per 10,000 input BM nucleated cells		
	BFU-E	CFU-Myeloid	CFU-Mix
1	2.3 ± 0.6	13.0 ± 3.8	2.5 ± 0.4
2	NA	NA	NA
3	8.7 ± 1.5	34.7 ± 11.2	1.7 ± 0.6
4	7.6 ± 1.4	26.1 ± 4.0	1.7 ± 0.2
5	5.0 ± 1.4	31.0 ± 1.4	0.5 ± 0.7
6	6.3 ± 0.1	16.3 ± 0.4	1.6 ± 0.3
7	6.5 ± 0.7	33.0 ± 4.2	1.5 ± 0.7
8	NA	NA	NA
9	NA	NA	NA
10	5.6 ± 0.6	15.4 ± 2.0	1.8 ± 0.3

Data shown are the number ± SD of human CFU-Cs per 10,000 input BM nucleated cells. The BM was obtained from NSG mice transplanted with AGM region and yolk sac cells. The recipient identification numbers are the same as in Table II. NA, not assessed, as the recipients died.

very limited self-renewal capacity by HSCs from the human UCB and adult BM (Guenechea et al., 2001; McKenzie et al., 2006; Liu et al., 2010). Although the microenvironment in immunodeficient mice is not optimal for the homeostatic expansion of human HSCs (Manz, 2007), human AGM region–derived HSCs extensively self-renew and amplify in these suboptimal conditions, suggesting that their expansion potential in physiological conditions is likely to be higher.

Extensive dissemination of daughter HSCs in primary recipients

The engraftment of one or two HSCs from the early human embryo results in considerable amplification of the HSC pool. We then tested if daughter HSCs stay at one site where the lodging of the parental HSC had occurred or if they migrate and disseminate throughout the recipient BM. We used two primary recipients repopulated with different AGM regions. We harvested BM from each recipient from the following six bones: two coxal bones, two femurs, and two tibiae (Fig. 5 A, mouse c). BM cell suspensions from each bone were transplanted separately into secondary recipients (Fig. 5 A, mice 1–6). In experiment 1, all secondary recipients showed human long-term multilineage hematopoietic engraftment (Fig. 5 B). Consistent with this, in experiment 2, all secondary recipients except one were repopulated with human hematopoietic cells. Thus, daughter HSCs generated by one or two human AGM region–derived HSCs disseminate broadly throughout the entire recipient BM.

Daughter HSCs are of the canonical phenotype

Using conventional FACS, it is not technically feasible to determine the immunophenotype of one or two HSCs present in one human AGM region. However, robust amplification of early embryonic HSCs in primary recipients allowed us to determine the immunophenotype of daughter HSCs. The BM harvested from the sternum, humeri, ulnae,

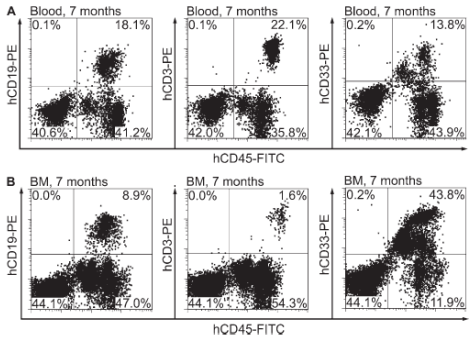

Figure 3. Human AGM region–derived HSCs provide long-term multilineage hematopoietic engraftment upon retransplantation into secondary recipients. Representative flow cytometry plots show human B ($CD19^+$), T ($CD3^+$), and myeloid cells ($CD33^+$) in the peripheral blood (A) and BM (B) of an NSG mouse transplanted with BM from a primary recipient engrafted with HSCs from a CS 15 AGM region. Secondary transplantation was performed 7 mo after primary transplantation. The analysis of secondary recipients was performed 7 mo later. Eight independent secondary transplantation experiments were performed.

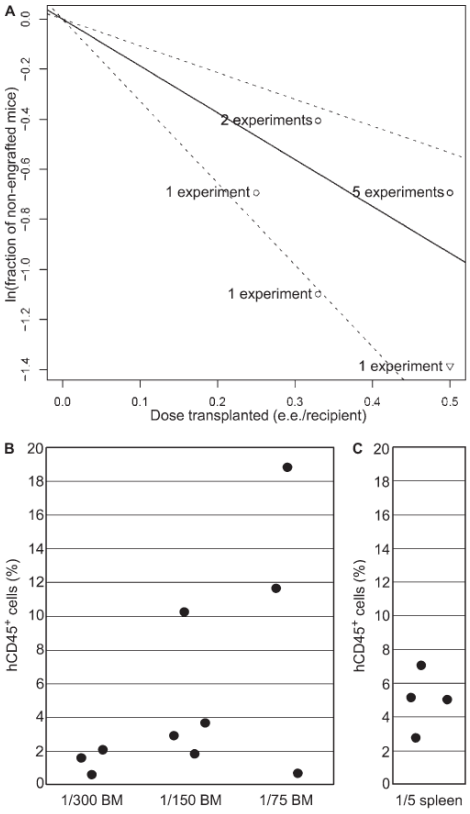

Figure 4. Extensive amplification of human AGM region–derived HSCs in primary recipients. (A) Results of 10 independent experiments in which HSCs were detected in AGM regions are plotted as the natural logarithm of the fraction of nonengrafted mice versus the dose of donor tissues (e.e. per recipient) transplanted in each experiment. The solid line shows the mean value. The dotted lines indicate 95% confidence interval. The data value with zero negative response (two out of two recipients were found engrafted with human HSCs) is represented by a down-pointing triangle. (B and C) 8 mo after transplantation, BM (B) and spleen (C) from a primary recipient repopulated with a single HSC from the AGM region of a CS 16 embryo were harvested and transplanted into secondary recipients (two independent experiments). Peripheral blood of secondary recipients was analyzed for human $CD45^+$ cell contribution 2 mo after transplantation. The ratios below the charts indicate the fraction of total BM or spleen transplanted per recipient.

radii, coxal bones, femurs, and tibiae of primary recipients engrafted with a single HSC from the human AGM region was sorted into four cell populations based on expression of human CD34 and CD38 antigens, and then transplanted into secondary recipients (Fig. 6 A). In two independent experiments, human HSCs were found exclusively within $CD34^{+}CD38^{-/lo}$ cell fraction (Fig. 6 B). Thus, HSCs derived from the early human embryo generate numerous HSCs of the canonical phenotype (Bhatia et al., 1997; McKenzie et al., 2006).

The human placenta develops HSCs later than the AGM region

In 19 independent experiments, we transplanted irradiated NSG mice with CS 12–17 placental cells and observed no long-term multilineage engraftment upon transplantation. Instead, in seven cases, placenta provided transient unilineage T cell repopulation of maternal origin (Table I, Table S5, and not depicted). The placenta contains maternal blood, and T cells from human blood can readily expand in immunodeficient mice (van Rijn et al., 2003). An older 15-wk-old placenta readily provided long-term multilineage hematopoietic repopulation of fetal origin in all three recipients (each received ~1/30 of total placental cells; Table S6 and not depicted). Thus, in contrast to the early CS 12–17 placenta, the midgestation human placenta does possess HSC activity. In the mouse embryo, definitive HSCs appear in the placenta concomitantly with the AGM region (Gekas et al., 2005; Ottersbach and Dzierzak, 2005).

DISCUSSION

Although the embryonic development of HSCs has been studied in various vertebrate species (Medvinsky et al., 2011), the generation of the first HSCs during human embryogenesis remains a largely unexplored topic. The present concept of the embryonic development of the human hematopoietic system is primarily based on morphological and in vitro studies (Tavian et al., 1996, 1999, 2001; Oberlin et al., 2002). By using functional in vivo long-term repopulation assays, we conducted systematic analysis of the spatiotemporal distribution of HSCs in the early human embryo. We have shown that the AGM region, more specifically the dorsal aorta, is the first generator of HSCs in the human. Interestingly, although in the mouse embryo definitive HSCs appear concomitantly in the AGM region, yolk sac, umbilical cord, and placenta (Müller et al., 1994; de Bruijn et al., 2000; Kumaravelu et al., 2002; Gekas et al., 2005; Ottersbach and Dzierzak, 2005), in the human embryo, the timing of HSC appearance in these sites is clearly

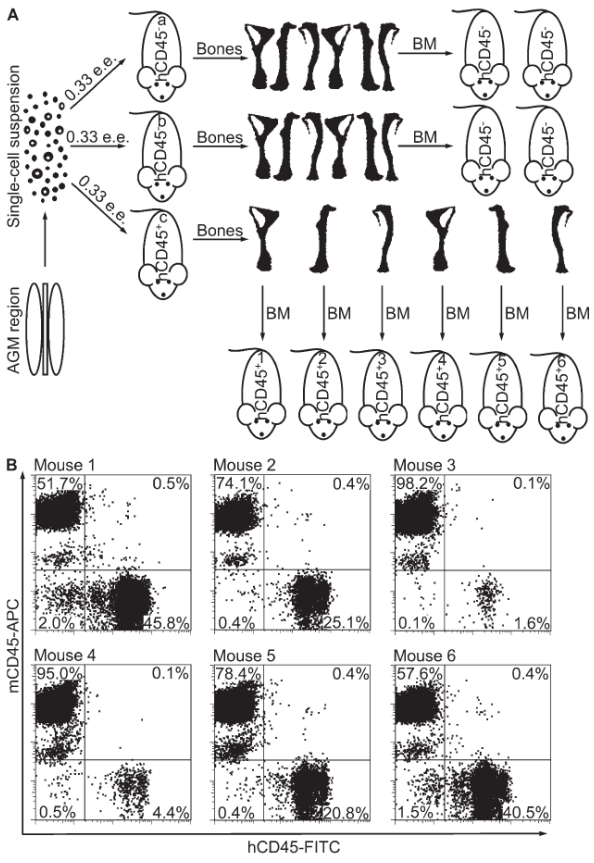

Figure 5. Extensive recipient BM colonization by daughter HSCs generated from a single AGM region–derived HSC. (A) AGM region cells obtained from a CS 15 embryo were transplanted into three NSG mice (0.33 e.e. per recipient). Only one of the three recipients showed human hematopoietic repopulation (mouse c). We confirmed by secondary transplantation that the other two recipients (mice a and b) contained no activatable HSCs. To test if the single human HSC that repopulated mouse c had generated daughter HSCs which could spread across the recipient BM, BM cells from two coxal bones, two femurs, and two tibiae were harvested and separately transplanted into six secondary recipients (mice 1–6). Secondary transplantations were performed 4 mo after the primary transplantation. (B) Representative flow cytometry plots show human hematopoietic repopulation in the peripheral blood of the six secondary recipients 3 mo later. Two independent experiments were performed. mCD45, mouse CD45.

resolved, possibly because of considerably longer development of the human embryo.

Our data indicate that early embryonic HSCs possess a considerably higher self-renewal capacity compared with UCB and adult BM HSCs (Guenechea et al., 2001; McKenzie et al., 2006; Liu et al., 2010). Upon transplantation into NSG mice, a single HSC from the human AGM region produces at least 300 daughter HSCs during a period of 8 mo; this unprecedented self-renewal capacity has not been previously described for human or for mouse AGM region–derived HSCs. It is likely because of this that the percentage of human leukocytes in the recipients' peripheral blood progressively increased, reaching up to 90% of total blood leukocytes. To achieve the same effect with UCB or adult BM, considerably higher numbers of HSCs need to be transplanted (Liu et al., 2010). More recent data suggest that ∼40% blood chimerism can be achieved 8 mo after transplantation of 10–20 UCB HSCs per one NSG mouse (Notta et al., 2011).

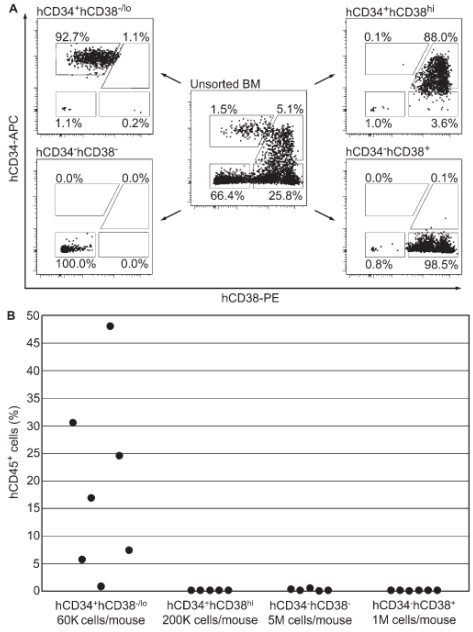

Figure 6. Daughter human AGM region–derived HSCs in primary recipients are CD34+CD38−/lo. (A) 7 mo after primary transplantation, BM of NSG mice repopulated with CS 16 and 17 AGM regions was sorted into four cell populations based on expression of human CD34 and CD38 antigens (two independent experiments). (B) Cells of each sorted population were injected into secondary recipients as indicated, and peripheral blood was analyzed 2 mo later. Two independent experiments were performed. K, multiple of thousand; M, multiple of million.

Using stochastic analyses, it has been shown that adult human HSCs replicate every 40 wk (Catlin et al., 2011). Our findings indicate that the extensive expansion of HSCs from the early human embryo occurs through replication every 3.5–4 wk or even more frequently, considering that HSCs also produce mature blood cells. This enormous regenerative capacity of early HSCs is consistent with them being founders of the adult hematopoietic system in the human.

We have shown that within the human AGM region HSCs emerge in the dorsal aorta. According to current views, it is possible that HSCs emerge in intraaortic cell clusters budding from the floor of the dorsal aorta (Jaffredo et al., 1998; Taoudi et al., 2008; Yokomizo and Dzierzak, 2010). In human, formation of such $CD34^+CD45^+$ cell clusters occurs between CS 12 and 16 (Tavian et al., 1996, 1999). Using limiting dilution analysis, we have shown that one human AGM region contains between one and three HSCs; however, the number of intraaortic cell clusters is much higher (Tavian et al., 1996, 1999), suggesting that not every cluster contains HSCs. Furthermore, although HSCs are still detected at CS 17, intraaortic cell clusters are reported to disappear by that stage (Tavian et al., 1996, 1999), indicating that the link between HSCs and hematopoietic cell clusters needs to be elucidated further. Notably, the maturation of definitive HSCs in the mouse embryo also occurs underneath the luminal layer of the dorsal aorta (Rybtsov et al., 2011).

As expected, HSC activity in the AGM region precedes that in the liver, which emerges there only after CS 17. Our data highlight the importance of in vivo long-term repopulation assays, as previous immunohistological and in vitro studies have suggested that HSCs appear in the human embryonic liver around CS 13 (Tavian et al., 1999). Recent transplantation experiments have shown that HSCs colonize the human embryonic liver around week 7–8 of development, which follows the stages we studied (Oberlin et al., 2010).

We demonstrate that in the human, in contrast to the mouse (Gekas et al., 2005; Ottersbach and Dzierzak, 2005), placenta acquires HSC activity long after the emergence of HSCs in the AGM region, indicating that in the human, placenta might be a secondary site for HSCs. Our data vary from a recent study's observation that the human placenta harbors HSCs from week 6 of development (as postmenstrual gestational age was employed, it is comparable with CS 12 or 13; Robin et al., 2009). In that study, NOD.CB17-*Prkdc*^{scid} (NOD/SCID) recipient mice, which are less receptive to human HSCs than NSG mice (McDermott et al., 2010), were used. The authors used a highly sensitive PCR analysis to detect repopulation with 6–8-wk-old placenta, identifying one human cell in 100,000 mouse cells. In the absence of flow cytometry analysis, a persistence of small numbers of some placenta-derived nonhematopoietic cells in recipients could not be excluded. However, the presence of multipotent HSCs in the placenta was convincingly shown by flow cytometry starting from week 9 of development (Robin et al., 2009).

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In summary, we provide a systematic spatiotemporal picture of HSC emergence in the early human embryo and identify the AGM region as the primary source of HSCs with unprecedented self-renewal capacity. Future comparative studies of early human embryonic HSCs and their fetal, neonatal, and adult counterparts may shed light on the mechanisms of HSC self-renewal and aging.

MATERIALS AND METHODS

Human embryonic and fetal tissues. Human embryonic and fetal tissues were obtained immediately after elective termination of pregnancy. The procedure was performed using mifepristone and misoprostol. The study was approved by the Lothian Research Ethics Committee. Each patient gave informed consent in writing. The developmental stage of human embryos was determined according to the Carnegie staging system (O'Rahilly and Müller, 1987). The postmenstrual gestational age of human fetuses was determined using medical ultrasonography. Tissue dissection was performed in buffer 1 (PBS with Ca^{2+} and Mg^{2+} [Sigma-Aldrich] supplemented with 7% heat-inactivated FBS [PAA Laboratories], 100 IU/ml of penicillin [Invitrogen], and 100 µg/ml of streptomycin [Invitrogen]). The dissection procedure was similar to that previously described for the mouse embryo (Medvinsky et al., 2008). For the enzymatic treatment of the human AGM region, yolk sac, liver, and umbilical cord, 1 mg/ml of collagenase/dispase (Roche), and 0.12 mg/ml of DNase I (Roche) were added to buffer 1 and incubated at 37°C for 40 min. For the enzymatic treatment of placental tissue, two previously published protocols were employed (Bárcena et al., 2009; Robin et al., 2009). In the majority of experiments with the human placenta, we omitted the Ficoll-Paque density gradient separation step to minimize cell losses. By the end of enzymatic treatment, tissue digests were washed with buffer 2 which, compared with buffer 1, was made with Ca^{2+} and Mg^{2+}-free PBS. For preparation of placental cells, in contrast to other tissues, 2 mM of EDTA was added to this buffer. After the first wash with buffer 2, tissue digests were pipetted gently to prepare a single-cell suspension. Human AGM region, yolk sac, liver, and umbilical cord cells were washed an additional time with buffer 2. Placental cells were passed through a 40-µm nylon cell strainer (BD) and washed four more times. Finally, cells were resuspended in buffer 2 and kept on ice until transplantation. Throughout the manuscript "independent" refers to those experiments performed with different human embryos.

Long-term repopulation assays. NSG mice were used as recipients for human HSCs. Animals were housed and bred within the University of Edinburgh, Edinburgh, Scotland, UK. Experiments with animals were approved by the Animal Welfare Committee of the University of Edinburgh and were performed according to the provisions of the Animal (Scientific Procedures) Act 1986 under the project license granted by the Home Office. All researchers who performed experiments with the mice held personal licenses granted by the Home Office. Mice were kept under specific pathogen–free conditions. Acidified (pH 3.0) autoclaved water supplemented with 1.67 mg/ml of neomycin (Invitrogen) and γ-irradiated chow diet was provided ad libitum. Animals were exposed to 14-h light and 10-h dark cycle. Up to 6 h before transplantation with human cells, 6–8-wk-old female NSG mice received a sublethal total body irradiation dose of 3.5 Gy at a rate of 0.75 Gy/min from a ^{137}Cs source (GSR D1 γ-irradiator; Gamma-Service Medical). Animals were transplanted with cells intravenously via the tail vein. The number of transplanted embryonic cells was expressed in embryo equivalents (e.e.), defined as a unit of cells equivalent to the number present in one tissue. Starting from 6–8 wk after transplantation, NSG mice were bled by tail vein nicking every 1–2 mo, and human HSC contribution was assessed by flow cytometry analysis after labeling cells with anti–human CD45 antibody. At the end of each experiment (usually 4–8 mo after transplantation), all recipients (including those found nonengrafted after peripheral blood analysis) were killed by dislocation of the neck according to the Schedule 1 of the Animal (Scientific Procedures) Act 1986, and their BM was collected. BM cells were labeled with anti–human CD45 antibody, and flow cytometry analysis was performed to detect the presence of human hematopoietic cells. The peripheral blood, BM, spleen, and thymus of engrafted NSG mice were further analyzed by flow cytometry analysis. The BM and spleen from primary recipients were used in secondary transplantation experiments.

Recipient mouse tissues. To collect recipient mouse tissues, buffer 2 supplemented with 2 mM of EDTA was used. NSG mice transplanted with human AGM region, yolk sac, liver, umbilical cord, placental cells, or BM from primary recipients were bled by tail vein nicking, and erythrocytes were lysed using Pharm Lyse Buffer (BD) according to the manufacturer's instructions. To analyze hematopoietic tissues, the spleen, thymus, long bones, coxal bones, and sternum were obtained. Spleen and thymus were gently mashed in a small volume of the buffer through a 40-µm nylon cell strainer. The BM was flushed out from the bones, and a single-cell suspension was prepared by gentle pipetting. A cell fraction associated with endosteum was obtained from remaining bones after treatment with collagenase/dispase and DNase I at 37°C for 40 min and added to the flushed-out BM fraction. The cells were spun down, resuspended in buffer 2 and kept on ice until transplantation. To calculate the proportion of total BM transplanted into secondary recipients, we used previously reported data on the distribution of BM cells in different mouse bones (Boggs, 1984).

Human CFU-C assay. Human CFU-Cs in the BM of NSG recipient mice were detected by plating in triplicates 10,000–25,000 BM nucleated cells in the MethoCult H4034 Optimum Medium (StemCell Technologies) according to the manufacturer's recommendations. The BM from a nontransplanted NSG mouse was used as a negative control. Cells were incubated at 37°C in 5% CO_2 and ≥95% humidity for 14 d. Hematopoietic colonies were enumerated microscopically. Some individual colonies were subject to flow cytometry analysis after labeling hematopoietic cells with anti–human CD45 and CD235a and anti–mouse CD45 and Ter119 antibodies.

Antibodies, flow cytometry analysis, and FACS. The following mouse anti–human monoclonal antibodies (all from BD) were used for flow cytometry analysis and/or FACS: CD3-APC, PE, and PerCP (clones SK7 and SP34-2), CD4-APC and APC-Cy7 (clone RPA-T4), CD8-PE and PE-Cy7 (clone RPA-T8), CD11b-PE-Cy7 (clone ICRF44), CD13-APC (clone WM15), CD14-APC and APC-Cy7 (clones M5E2 and MφP9), CD19-PE (clone HIB19), CD33-PE (clone WM53), CD34-APC (clone 8G12), CD38-PE (clone HIT2), CD41a-FITC (HIP8), CD45-Biotin, FITC, and V450 (clone HI30), CD66b-FITC (clone G10F5), CD94-APC (clone HP-3D9), CD235a-APC (clone GA-R2), IgM-Biotin (clone G20-127), αβ TCR-FITC (clone WT31), γδ TCR-PE (clone 11F2). Appropriate isotype controls, streptavidin-APC, rat anti–mouse CD45-APC (clone 30-F11), and Ter119-PE and FITC were also purchased from BD. The Human FcR Blocking Reagent (Miltenyi Biotec) and anti–mouse CD16/32 purified monoclonal antibody (clone 93; eBioscience) were used to prevent unwanted binding of antibodies to Fc receptors. All of the aforementioned antibodies and reagents were used at final concentrations recommended by manufacturers or determined by in-house titration. Cell labeling and washes were performed in Ca^{2+} and Mg^{2+}-free PBS supplemented with 2% heat-inactivated FBS. For dead cell exclusion the buffer was supplemented with 1.5 µg/ml of 7-amino-actinomycin (eBioscience). A FACSCalibur (BD), LSRFortessa (BD), or CyAn ADP (Dako) instruments were used for flow cytometry analysis. FACS and postsort purity check were performed on a FACSAria II instrument (BD). Sorted cells were spun down, resuspended in buffer 2, and kept on ice until transplantation. Flow cytometry data were analyzed with FlowJo v7.6.1 software (Tree Star).

STR analysis. Genomic DNA was extracted from peripheral blood, spleen, and/or BM cells obtained from NSG mice repopulated with human hematopoietic cells and from embryonic or fetal donor tissues. For this, the QIAamp DNA Investigator Kit (QIAGEN) and a QIAcube robotic instrument (QIAGEN) were used. DNA quantification was performed with the Plexor HY System (Promega) and an Mx3500P real-time PCR machine (Stratagene).

DNA quantification values were determined using the Plexor Data Analysis Software (Promega). DNA samples and PCR positive and negative controls were amplified with the AmpFlSTR SGM Plus PCR Amplification Kit (Applied Biosystems). PCR products were detected on a 3130 Genetic Analyzer (Applied Biosystems). Handling of raw data and genotyping were performed with GeneMapper ID v3.2.1 software (Applied Biosystems). All the reagents and instruments listed above were used according to manufacturers' recommendations. DNA match probabilities were calculated based on allele frequencies in Scottish Caucasian population.

Limiting dilution analysis. Limiting dilution analysis was performed using ELDA software (Hu and Smyth, 2009) available at http://bioinf.wehi.edu.au/software/elda/.

Online supplemental material. Tables S1–S6 show STR analysis data confirming either embryonic or maternal origin of hematopoietic repopulation in recipients transplanted with human embryo or placenta-derived cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20111688/DC1.

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Author contributions: A. Ivanovs performed the majority of transplantation experiments, interpreted experimental data, and wrote the manuscript; S. Rybtsov performed some transplantation experiments and interpreted experimental data; L. Welch performed STR analysis and interpreted its data; R.A. Anderson and M.L. Turner interpreted experimental data; and A. Medvisnky directed the study, interpreted experimental data, and wrote the manuscript.

The authors declare no competing financial interests.

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